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Award Number: DAMD17-95-1-5017

TITLE: Role of Tumor Collagenase Stimulating Factor in Breast Cancer Invasion and Metastasis

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REPORT DATE: December 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	December 1999	Final (1 Dec 94 - 30 Nov 99)

4. TITLE AND SUBTITLE
Role of Tumor Collagenase Stimulating Factor in Breast Cancer Invasion and Metastasis

5. FUNDING NUMBERS
DAMD17-95-1-5017

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Extracellular matrix metalloproteinase inhibitor (EMMPRIN), a plasma membrane glycoprotein identified originally in carcinoma cells, is responsible for inducing peritumoral fibroblasts to produce matrix metalloproteinases (MMPs), thereby enhancing cancer invasion and metastasis. Using in situ hybridization and immunohistochemistry, we have identified EMMPRIN in breast cancer tissue. We have characterized the human EMMPRIN gene and have noted a high degree of conservation with the mouse gene. The effect of transfecting human breast cancer cells with EMMPRIN cDNA was explored. Expression of EMMPRIN cDNA by MDA-MB-436 cancer cells resulted in marked increase in tumor growth and invasiveness after tumor cell injection into mammary tissue of nude mice. Using the phage display system, crosslinking and ligand affinity chromatography, MMP-1 binding to EMMPRIN was identified suggesting the novel possibility that EMMPRIN binds MMP-1 to the tumor cell surface, thus enhancing pericellular degradation of extracellular matrix. The effect of EMMPRIN on endothelial MMPs was explored in vitro; EMMPRIN enhanced endothelial production of stromelysin-1, collagenase, and gelatinase A. These data support a role for EMMPRIN in enhancing cancer dissemination.

14. SUBJECT TERMS Breast Cancer
Metastasis, Metalloproteinases, Cytokines, Collagenase, Fibroblasts, Mutations, Humans, Anatomic Samples

15. NUMBER OF PAGES 126

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Stanley Zucker 3/7/00

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(5) INTRODUCTION

The purpose of this grant has been to determine the role of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN) in breast cancer invasion and metastasis. EMMPRIN is a glycoprotein identified on the plasma membrane of cancer cells which induces fibroblasts to produce Matrix Metalloproteinases (MMPs) (1).Originally known as Tumor Collagenase Stimulating Factor (TCSF) because it was described as a stimulator of interstitial collagenase (MMP-1) production by fibroblasts, TCSF was subsequently demonstrated to also induce target cells to synthesize gelatinase A and stromelysin-1, hence the name change to EMMPRIN to reflect induction of synthesis of other MMPs.

EMMPRIN (TCSF), was initially purified from the plasma membranes of cancer cells by Biswas et al. and identified as a 58 kDa glycoprotein. EMMPRIN had no mitogenic activity and therefore differs from most well characterized cytokines, such as IL-1, TNF, and TGF-β. Monoclonal antibodies raised against EMMPRIN were used to purify and characterize this membrane glycoprotein (2). The E11F4 monoclonal antibody also inhibited the biological activity of EMMPRIN, thereby proving that EMMPRIN was the effector molecule. Following development of specific monoclonal antibodies, EMMPRIN was identified by immunohistochemistry in the cell membranes of malignant epithelial cells from tumor specimens of human lung cancer and bladder cancer (3). Recent reports from other laboratories have confirmed the stimulatory effect of human breast and bladder cancer cells on production of MMPs by host fibroblasts (3, 4).

The main thesis of this grant is that breast cancer cells produce EMMPRIN which then induces peri-tumoral fibroblasts to produce the MMPs (collagenase, gelatinase A, and stromelysin-1) required for cancer dissemination. The biologic role of EMMPRIN in cancer was not initially appreciated because tumor cell lines grown in vitro generally produce high levels of MMPs, thereby suggesting that tumor cell production of MMPs was responsible for cancer invasion and metastasis. Fibroblast production of MMPs was relegated to a secondary role. In retrospect, this confusion seems to have been brought about because cancer cells that develop into immortalized cells in tissue culture generally produce high levels of MMPs. Cancer cells incapable of producing MMPs in vitro seem to be at a disadvantage in terms of developing into immortalized cell lines. Based on these observations, we postulate that high MMP production is a selection factor for enhanced cell growth in vitro. Hence, although the vast majority of cancer cells within a tumor do not produce MMPs in high levels, the few cells that produce MMPs appear to have a growth advantage in tissue culture. These data may explain the lack of enthusiasm for a role for EMMPRIN in cancer dissemination until it was demonstrated that the MMP producing cells within a tumor are primarily host fibroblasts rather than cancer cells.

Considerable evidence has been presented in the past 4 years to support the Biswas concept that cancer cells signal fibroblast to produce MMPs (1). In situ hybridization studies of human breast cancer tissues have shown that the cDNA for matrix metalloproteinases (stromelysin-3, gelatinase A, interstitial collagenase, MT-MMP) is localized to fibroblasts surrounding the tumor rather than to the tumor cells themselves (5, 6). Immunohistochemical studies using monoclonal and polyclonal antibodies, however, have identified gelatinase A in breast cancer cells suggesting that the tumor cells may bind this MMP to their cell surface (7-9). Interpretation of these data has led to the conclusion that normal host fibroblasts produce much of the MMPs that the cancer cells utilizes during invasion (10).

Using immunohistochemistry, we have demonstrated the selective localization of EMMPRIN on the surface of malignant cells in human breast cancer tissue (11), further suggesting that this factor may provide the missing link to explain the observation that peritumoral fibroblasts are the major producers of MMPs.

Based of the potential importance of EMMPRIN in regulation of MMP activity during tumor cell invasion, we have studied EMMPRIN at the molecular and physiologic level. Following determination of the nucleotide sequence of the cDNA for human EMMPRIN (1), it

was recognized that EMMPRIN is homologous to proteins of the Ig superfamily (basigin, neurothelin, OX-47, M-6) (12, 13) which have been identified in arthritis and embryonic epithelial/stromal interactions; the function of these proteins was not explored in these latter studies, but a function similar to EMMPRIN would be appropriate in these situations.

(6) BODY OF ANNUAL REPORT

Experimental Results (the original timetable for these tasks is listed in parentheses)
Accomplishments achieved in years 1-3 (12/94 to 11/97), year 4 and extension year 5 (12/97 to 11/99) are identified separately.

Task (1). Identify the cellular localization of EMMPRIN (TCSF) and MMPs in human breast cancer tissue.

(1a) Obtain tissue samples from patients with various forms of breast cancer (24 months):

12/94 to 11/97- Breast tissue samples were obtained from 87 women with various forms of breast cancer and women with benign breast disease.

12/97 to 11/99- Breast tissue samples were obtained from an additional 12 patients. This number of samples has been sufficient to perform the experiments initially planned.

(1b) Immunolocalization of EMMPRIN and MMPs in human breast cancer tissue using specific antibodies to EMMPRIN to determine epithelial:mesenchymal contributions (36 months):

12/94 to 11/97- Using immunohistochemical techniques and our original E11F4 monoclonal antibody to EMMPRIN, we characterized the cellular localization of EMMPRIN as compared to the localization of gelatinase A in breast cancer. Tumor sections obtained from 28 women with breast cancer were examined. In all cases of invasive ductal cancer, antibodies to EMMPRIN reacted strongly with invasive cancer cells with intense staining of the plasma membrane and less intense staining of cytoplasm. In comparison with cancer cells, normal ducts within the tumor specimen demonstrated similar staining with anti-EMMPRIN antibody. EMMPRIN immunostaining was intense in both early and advanced stages of invasive breast cancer, as well as in situ breast carcinomas. Moderate EMMPRIN staining of breast ducts and acini was noted in breast tissue obtained from biopsies of patients with benign breast disease and normal breast tissue (obtained from mammary reduction surgery). Colored photos were displayed in the 1996 report.

Limitations in quantifying EMMPRIN antigen concentrations in human tissue using the E11F4 monoclonal antibody could not be ruled out. These data suggested that EMMPRIN may have a function in embryonic development or maintenance of normal breast tissue, as well as the malignant process. We propose that in physiologic processes, the presence of an intact basement membrane separating the normal/benign epithelium from underlying stromal fibroblasts limits access of epithelial cell EMMPRIN for induction of MMP production by stromal fibroblasts through a cell-cell contact related mechanism. In contrast, in carcinomas the epithelial basement membrane is fragmented, thereby permitting epithelial cancer cells to migrate into the stroma, make direct cell contact with fibroblasts, and stimulate fibroblast synthesis of MMPs. The enhanced production of MMPs by peritumoral fibroblasts then leads to degradation of the stroma (including the basement membrane), thereby enhancing the invasive/metastatic process of cancer cells.

12/97 to 11/99- The discrepancy between our results on immunohistochemistry and in situ hybridization (see below) have led us to repeat our earlier immunohistochemical studies using a different monoclonal antibody to EMMPRIN. In this study (J. Histochemistry and Cytochem. 47: 1575-1580, 1999), we used a recently developed mouse monoclonal antibody directed against EMMPRIN (see below) for immunohistochemical localization of EMMPRIN. Fresh frozen tissue from women with breast cancer and specimens of fibroadenoma of the breast were examined. EMMPRIN protein was prominently displayed in malignant breast tissue on both the surface of tumor epithelial cells and fibroblast-like stromal cells; occasional endothelial cells displayed spotty labeling on their cell membranes. In carcinomas there were also fine positive vesicles in the connective tissue close to tumor cells suggesting that EMMPRIN could be released as membrane components by tumor cells. EMMPRIN was frequently confined to the apical cell membrane. In normal or benign cells, EMMPRIN was also detected as a weak label at the basal pole of the epithelial cells. In tumor clusters,

EMMPRIN was highly expressed in all malignant cells with more intense staining on the cells located at the periphery of well differentiated tumor nests (Figure submitted in 1998). Colocalization of EMMPRIN with MMP-2 was examined. In all carcinomas, we observed that the presence of EMMPRIN in/on stromal cells was concomitant with the detection of MMP-2 in the same cells. Using double labeling of immunofluorescence, we found that the stromal positive staining of EMMPRIN was not necessarily associated with the absence of type IV collagen around tumor clusters suggesting that basement membrane integrity is not a limiting factor for EMMPRIN diffusion from the producing cell. Since previous studies have demonstrated that stromal cells did not express EMMPRIN mRNAs, it is very likely that EMMPRIN is release or shed as vesicles from the membranes of cancer cells and then binds to stromal cells via a receptor. Taken together, our results showed that EMMPRIN is an important factor in tumor progression by causing tumor-associated stromal cells to increase their MMP production, thus facilitating tumor invasion and neoangiogenesis.

(1.b.1) Production of monoclonal antibodies to EMMPRIN

12/94 to 11/97- Production of monoclonal and polyclonal antibodies to recombinant EMMPRIN for use in ELISAs. Eight BALB/c mice were immunized by the intraperitoneal injection of recombinant EMMPRIN (purified from CHO cell homogenates transfected with EMMPRIN cDNA). In spite of the development of high serum titers of antibodies against the immunogen in mice, antibodies adequate for ELISA were not produced. The problem with the initial 20 mouse myeloma clones developed was that all of the antibodies were of the IgM type and reacted nonspecifically with other proteins as demonstrated by Western blotting.

12/97 to 11/99- To circumvent these problems, we used EMMPRIN purified from human lung cancer cells (LX-1) rather than recombinant EMMPRIN as the immunogen. EMMPRIN antigen injections (100 ug) in mice were performed on a 2 week schedule and mice were sacrificed after 8 weeks. This work was done in collaboration with Chemicon Corp. (Drs. Dale Dembro and Alex Strongin, San Diego, CA). Antibodies to EMMPRIN in mouse serum were present at a titer of 1:10,000 using native antigen rather than recombinant antigen. Spleen myeloma cells fusions growing in splenocyte conditioned media resulted in the production of 63 positive IgG producing wells. The 15 clones with the highest antibody titers as detected by EIA (20 ng EMMPRIN per well) were subcloned with 8 active clones identified after two subcloning procedures. All 8 clones produced distinct, broad bands (a result of extensive glycosylation) at ~53 kDa on Western blotting with EMMPRIN; a second band at 28 kDa representing either non glycosylated EMMPRIN or a degradation product of EMMPRIN was also noted. Non-specific cross reactivity with other proteins was not a problem. Two of these monoclonal antibodies have been characterized more fully and have been shown to be effective for Western blotting and immunohistochemistry. We have used these antibodies to screen for cells expressing high levels of EMMPRIN antigen on their plasma membranes.

(1.b.2) New Aim: Production of monoclonal antibodies to Membrane Type (MT1)-MMP

12/94 to 11/97- Since the description of MT1-MMP in 1994 (16, 17) , it has become apparent that this membrane bound MMP is important in cancer because it functions to activate progelatinase A in the pericellular environment. We propose that following the induction of progelatinase A synthesis by EMMPRIN, MT1-MMP is required for activation of progelatinase A. As with most MMPs, MT1-MMP has been described by in situ hybridization as being expressed in tumoral fibroblasts rather than epithelial cancer cells (18). Since our previous study demonstrated that EMMPRIN treated fibroblast secreted activated as well as latent gelatinase A (19), this data suggested that EMMPRIN may also be responsible for inducing the synthesis of MT1-MMP. Based on these observations we have developed high titer monoclonal antibodies that react with MT1-MMP by injecting recombinant human MT1-MMP (fusion protein with GST) lacking the transmembrane domain (protein produced in bacteria) into Balb/c mice. Following fusion of mouse spleen cells with immortalized myeloma cells, antibody producing clones were selected. Six of these monoclonal antibodies demonstrate high titer efficiency as detecting antibodies for immobilized MT1-MMP. In these

studies, we became aware that all of these monoclonal antibodies were reactive only with secreted forms of MT1-MMP lacking the transmembrane domain. These antibodies were not reactive with cell-bound MT1-MMP and hence, are not useful for immunochemistry or ELISAs of tissue homogenates. These antibodies, however, might be useful for identifying secreted/shed forms of MT1-MMP in body fluids. In the past 2 years, Chemicon International and Oncogene Sciences have commercially made available several antibodies to MT-MMPs. Therefore, the goal of this aim is no longer relevant and this aim has been terminated.

(1c) Develop an ELISA for EMMPRIN (TCSF) for use in quantifying antigen in breast tissue (6 months):

12/96 to 11/99- The development of an ELISA for EMMPRIN has not been successful because of the absence of antibodies of sufficiently high titer and affinity for EMMPRIN. Commercial antibodies to EMMPRIN have not been useful for developing an ELISA.

(1d) Quantify the EMMPRIN, gelatinase A, gelatinase B, and stromelysin-1 content of fresh tissue samples obtained from patients with breast cancer (36 months):

12/94 to 11/99- These studies have not been completed because we have not been successful in developing a method for quantifying the concentration of EMMPRIN in tissue samples. Our ELISA has not been useful for tissue assay of EMMPRIN. Because of our success in demonstrating increased EMMPRIN expression in cancer tissues by immunohistochemistry (task 1b) and in situ hybridization (task 1e), further efforts to complete this task are no longer considered to be of major importance; therefore this goal has been abandoned.

(1e) Identify mRNA for EMMPRIN (TCSF) in breast cancer tissue using in situ hybridization (48 months):

12/94 to 11/97- This task was completed ahead of schedule. To characterize and distinguish the cells producing EMMPRIN and gelatinase A in breast cancer, we employed in situ hybridization using radiolabeled RNA probes for EMMPRIN and gelatinase A (20). Surgical specimens were obtained from 22 women with breast cancer and from 7 women with benign breast disease (fibrocystic disease and fibroadenoma). The result of these studies was that EMMPRIN mRNA was detected by in situ hybridization in all carcinomas in both non invasive and invasive cancer cells and in pre malignant areas such as atypical hyperplasia of the breast. EMMPRIN mRNA and gelatinase A mRNA were both visualized in the same areas in serial sections in breast cancer, but were expressed by different cells with tumor cells expressing EMMPRIN mRNA and fibroblasts expressing gelatinase A mRNA. There was no correlation between EMMPRIN mRNA and the tumor size, grade of the tumors, the number of lymph node metastases, and the hormonal receptor status of the tumors. Normal mammary glands adjacent to cancer areas showed no EMMPRIN hybridization grains. By Northern blot analysis of tissue extracts, higher expression of EMMPRIN mRNAs in breast cancers was noted as compared to benign and normal breast tissue; however, the normal tissue expression was not negative as noted by in situ hybridization. The discrepancy noted between normal breast ducts staining positively for EMMPRIN by immunohistochemistry but negatively by in situ hybridization suggested differences in sensitivity of these techniques for EMMPRIN detection or differences in the rates of EMMPRIN turnover in normal versus malignant tissue (21).

Task (2). Identify important structural:functional relationships in the EMMPRIN (TCSF) molecule.

(2a) Determine whether post-translational processing is required for biological activity (12 months): **This task has been completed.**

12/96 to 11/97 - To circumvent problems related to the requirement for glycosylation of EMMPRIN for function of the protein, we stably transfected mammalian cells (CHO) with EMMPRIN cDNA. Posttranslational processing resulted in the production of EMMPRIN of

molecular weight identical to native EMMPRIN from tumor cells i.e. ~58 kDa. We immunopurified the recombinant EMMPRIN after extraction from CHO cell membranes using monoclonal antibodies raised against native tumor cell EMMPRIN. When added to human fibroblasts in culture, the purified recombinant EMMPRIN was found to be active in stimulating production (2-5 fold) of fibroblast interstitial collagenase, gelatinase A, and stromelysin-1, but not TIMP-1 (22). Since non-glycosylated and partially glycosylated recombinant EMMPRIN were unable to stimulate MMP production, we conclude that post-translational processing is required for EMMPRIN activity.

(2b) Alter EMMPRIN (TCSF) by deletional mutation and site directed mutagenesis of cDNA and then analyze mutant proteins to determine the minimum amino acid sequences necessary for functional activity (36 months):

(2.b.1) **12/94 to 11/97**- We reconfirmed the identity of the EMMPRIN cDNA by showing that recombinant protein is recognized by the activity blocking monoclonal antibody (E11F4). Two immunoreactive bands were obtained corresponding to the forms previously noted (1). The bacterial recombinant proteins was also used to determine the approximate location of the epitope for E11F4, taking advantage of the lack of posttranslational modification that would interfere with such studies on the native, immunoaffinity-purified protein. Modified EMMPRIN expression plasmids were made containing deletion in four locations. XL-1 blue cells were transformed with the deletions expression pBluescript plasmids, and the expressed proteins were analyzed by SDS-PAGE and Western blotting with monoclonal antibody E11F4. All of the plasmids produced protein that is immunoreactive, except for the plasmid lacking immunoglobulin domain I. These results demonstrate that our cDNA encodes the protein which is reactive with our activity-blocking monoclonal antibody and that the antibody epitope exists in the extracellular immunoglobulin domain I. This, in turn, implies that the functional site of the metalloproteinase stimulatory activity of EMMPRIN is likely to be localized to sequences contained in the immunoglobulin domain I region.

(2.b.2) Characterization of the human EMMPRIN gene

As a result of our sequencing of keratinocyte cDNA for EMMPRIN and its strong homology with cancer cell EMMPRIN, and the identification of EMMPRIN in normal rabbit kidney cells, T lymphocytes, and erythrocytes, we considered the strong possibility that the difference between the high level of synthesis of EMMPRIN in cancer cells and the apparent low level synthesis in non malignant cells may be due to differences in the promoter region for EMMPRIN, specifically in the mixed response element category. This led us to undertake the sequencing of the entire human EMMPRIN gene as a priority goal.

cDNA hybridization have been used by other investigators to determine the chromosomal location of mouse EMMPRIN/basigin gene, mapping to chromosome 10.

12/96 to 11/98- We have reported the characterization of the human EMMPRIN gene. Using EMMPRIN cDNA, we isolated a >30 kbase cosmid clone containing the human EMMPRIN gene and confirmed the gene localization to chromosome 19p13.3. Using a fragment of the clone, S1 was performed to determine the transcriptional start site. The exon/intron boundaries were determined by genomic PCRs and sequencing. The gene fragment contains ~ 1kBase of sequence upstream of the transcription start site. This 5' flanking region does not contain TATA or CAAT boxes (figures presented in 1997 report). However, the start site falls within a CpG island. Also three consensus binding sites for SP1 and two for SP2 were found (23).

Elements in the proximal promoter region were conserved in human and mouse genes. The human and mouse genes have the unusual property that each Ig domain is not encoded by one exon, but by two. Also unusual in the EMMPRIN gene as compared to other Ig family members is the fact that the downstream exon of the second Ig domain is a junctional exon encoding the transmembrane domain and part of the cytoplasmic domain as well. Most members of the Ig superfamily encode the Ig domain in a single, unshared exon.

These data lead us to postulate that when cells become transformed they might produce

more SP1 and AP2 and those are the factors which bind to the EMMPRIN promoter, thereby enhancing the transcription and subsequent translation of EMMPRIN.

(2c) Design peptide antagonists and produce anti-functional monoclonal antibodies to further characterize the structure:functional relationship of the EMMPRIN molecule (48 months):

This task was not able to be completed within the time frame of this study. We have produced several monoclonal antibodies to EMMPRIN, but none of these has been demonstrated to have anti-functional activity.

Further efforts to complete this task will be undertaken even though the current funding mechanism is no longer available.

Task 3. Explore the role of EMMPRIN (TCSF) in cancer dissemination using experimental models.

(3a) Compare the effect of transfecting breast cancer cells with cDNA for native versus mutant EMMPRIN in regards to altering cancer invasion and metastasis in an experimental model (48 months):

12/94 to 11/97- *Preliminary experiment:* The open reading frame for EMMPRIN cDNA was transfected into human breast cancer cell lines (MDA-MB-436 and MCF-7). Transfected cell lines were then injected into the mammary fat pad of 6-8 week old nude female mice at a cell concentration of 1 X 10^6 per animal. Palpable tumors (1-4 cm in diameter) were detected after 3 months in 2/4 mice injected with EMMPRIN cDNA- transfected MDA-MB-436 cells and in 3/4 mice with mock transfected MDA-MB-436 cells. Histology of the breast cancers was as anticipated indicating that the morphologic phenotype had not changed. This experiment was subsequently refined in 1997 as described below.

12/97 to 11/99- To ascertain that transfected tumor cells are expressing EMMPRIN protein in high concentration, we took advantage of the recent observation that the green fluorescent protein (GFP) of the jelly fish Aequoria victora retains its fluorescent properties when recombinantly expressed in eukaryotic cells (24). This 29 kDa protein can then be used as a powerful marker for gene expression in vivo (25). To this end, we expressed EMMPRIN cDNA along with the GFP reporter cDNA as a fusion gene controlled by a CMV promoter in pcDNA3 expression vector. The GFP-EMMPRIN fusion protein was expressed in the plasma membrane of transfected COS-1 cells as documented using fluorescent microscopy, thus indicating that GFP is transported to the plasma membrane along with the EMMPRIN protein. A second plasmid was produced in which GFP and EMMPRIN are controlled individually by separate CMV promoters. A control plasmid containing GFP alone was also produced (Figure 1). We documented EMMPRIN expression in transfected cells by fluorescent microscopy and immunoblotting of cell lysates (Figure 2A).

MDA-MB-436 human breast cancer cells were transfected with GFP cDNA and EMMPRIN/GFP fusion cDNA. Stable cell lines were selected using G-418. After 5 weeks, stably transfected cells were examined by Northern blotting. The expression of EMMPRIN mRNA in EMMPRIN transfected MDA-MB 436 cells was approximately 20 fold higher than in vector transfected cells (Figure 1B). 1 x 10^7 tumor cells derived from these transfected cell lines were then injected into mammary tissue of nude female mice. Mice were examined weekly thereafter for tumor size measurements.

Results: EMMPRIN/GFP transfected mice developed breast tumors at the site of mammary injections that grew to 1.4-2.3 cm diameter within 7-12 weeks; intra-abdominal metastases were extensive in most mice (retroperitoneal, perigastric, sub diaphragm, kidney, etc.). These tumors were highly vascularized with numerous grossly visible vessels entering the tumor mass from normal tissue. GFP expressing tumors were green in color when examined by fluorescent light. Lung metastases were not observed in any of the groups. By comparison, the GFP (only) transfected tumor cells produced small, slow growing, poorly vascularized tumors (3/4 mice)

that reached a diameter of ~2-3 mm in 12 weeks (not visible until autopsy).

12/97 to 11/99- These experiment have been repeated in order to determine the reproducibility of these observations. Groups of 10 nude female mice (4-6 weeks of age, younger than our 1997 experiment) have been injected with 1 X 10^6 transfected MDA-MB-436 cells (lower cell number) into mammary tissue as described above. Tumor formation and metastases were recorded during 15 weeks of observation. Major differences in groups were noted. The tumors derived from the EMMPRIN/GFP cDNA transfected MDA-MB-436 tumor cell injections grew much more rapidly and all 10 mice expired within 15 weeks; intra-abdominal metastases were frequent in these mice. In contrast, injection of the GFP cDNA alone transfected tumor cells into mice resulted in tumors that grew much more slowly; none of these mice expired and no intra-abdominal metastases were noted by termination of the experiment at week 15 (Figure 3). Blood vessels leading to the tumor were considerably more extensive on gross observation of mice in the EMMPRIN/GFP transfected MDA-MB-436 group (Figure 2B).

Gelatinolytic Activity Extracted from Tumor Tissues and Cells

Gelatin zymograms of conditioned media from cultivated MDA-MB-436 cells (Figure 3B) revealed that cells transfected with GFP or EMMPRIN/GFP cDNA secreted equivalent amounts of 72 kDa gelatinase A; gelatinase B was not detected. Tumor extracts from EMMPRIN/GFP injected mice displayed intense gelatinolytic bands localized at 105 kDa, 92 kDa, 72 kDa, and 64-62 kDa. The 105 kDa band is consistent with mouse latent gelatinase B; human latent gelatinase B migrates at 92 kDa. The 72 kDa and 62 kDa gelatinolytic bands could represent human or mouse latent and activated gelatinase A, respectively. Even though the protein content was equalized between specimens, tumor extracts from GFP alone-injected mice revealed weaker gelatinolytic bands than EMMPRIN/GFP injected mice.

Histochemistry/In situ Hybridization

Hematoxylin and eosin staining of resected breast masses revealed extensive replacement of normal mammary tissue with carcinoma in tumors originating from mice injected with EMMPRIN/GFP or GFP transfected MDA-MB-436 cells. In situ hybridization of tumor tissue from mice injected with EMMPRIN/GFP-transfected cells revealed widely distributed, specific staining with EMMPRIN in cancer cells; surrounding benign appearing mammary ducts also expressed EMMPRIN (Figure 4). Gelatinase A staining was found in both cancer cells and the surrounding non malignant tissue (muscle, fat, and benign-appearing mammary ducts). There was specific staining with the gelatinase B antisense riboprobe in cancer cells, but not as widely distributed as gelatinase A; intense periductal staining was noted in surrounding normal tissue. Similar results were found on examination of metastatic tumors in the EMMPRIN/GFP treated mice. Specific staining was abolished by pretreatment of tissues with RNAase (data not shown). In the GFP alone-transfected tumors, virtually no EMMPRIN or gelatinase A staining was seen. Focal staining for gelatinase B was noted in GFP tumor tissue, not in surrounding normal tissue. No staining was detected in any of the tumor tissues that were hybridized with EMMPRIN, gelatinase A, or gelatinase B sense probes. For details, see enclosed manuscript recently submitted for publication.

In summary, these experiments suggests that: 1) transfection with EMMPRIN cDNA resulted in more rapid tumor growth, intra-abdominal metastases, and extensive tumor neovascularization; and 2) in the background of this less malignant transfected phenotype, expression of EMMPRIN cDNA resulted in more invasive and locally metastatic cancer cells.

(3b) Analyze human peritumoral fibroblast response to EMMPRIN (TCSF) in vitro (36 months):

12/97-11/99

(3b1) In a collaborative study with Dr. Nabeshima et al. we have investigated the expression of EMMPRIN in human brain tumors, especially astrocytic tumors. Both normal

brain and astrocytic tumors expressed EMMPRIN at the mRNA (Northern blotting) and protein (immunoblotting) levels, but expression levels were higher especially in high grade astrocytic tumors. Localization was quite different between normal and tumor tissue. In the normal brain only vascular endothelial cells were EMMPRIN-positive, whereas in the tumors the cancer cells were positive and the endothelium was negative (26). EMMPRIN positivity in endothelium was present only in the brain which is consistent with localization in the blood brain barrier as previously reported for Basigin (murine EMMPRIN) in mice.

In vitro co-culture of EMMPRIN-positive glioblastoma cells and brain-derived fibroblasts resulted in increased gelatinase A production as compared with control, and this stimulatory effect was inhibited by anti-functional anti-EMMPRIN antibody (EIIF4). This data is consistent with the involvement of EMMPRIN in tumor invasion and angiogenesis since brain-derived fibroblasts are present in perivascular tissue (26).

(3b2) Evaluate more directly the mechanism of action of EMMPRIN in terms of cell surface receptors, intracellular messengers, and mechanism of inducing MMP gene expression.

Based on additional information available about EMMPRIN since 1994, it would appear that identifying the receptor for EMMPRIN on fibroblast target cells is a priority in understanding the mechanism of action of EMMPRIN and in determining whether tumor derived fibroblasts are more responsive to EMMPRIN's stimulatory effect than non activated fibroblasts. Based on these considerations, we have performed receptor binding studies with EMMPRIN. Cross linking- Human HFL (lung fibroblasts) were metabolically labeled with ^{35}S methionine (200 uCi/ml) for 5 hours. Cells were washed thoroughly to remove unincorporated isotope. Purified human EMMPRIN (100 nM) was added to cells for 1.5 hours. The cross-linking reagent, 0.5 mM DTSSP (Pierce Co.) was added for 30 minutes at 23° C. The cells were then washed thoroughly and cells were lysed in PBS buffer with 0.1% triton X-100 detergent. The cell lysate was incubated with anti-EMMPRIN antibody (E11F4) overnight and then added to protein A agarose beads for 1 hour. The beads were then washed six times with PBS and boiled with 1x sample buffer for 5 minutes. The samples were loaded on a 10% SDS-PAGE and exposed to XRay film. The results demonstrate the binding of EMMPRIN to a ~67 kDa and a ~55 kDa protein on fibroblasts (Figure 5).

We next examined the binding of EMMPRIN to fibroblast membranes. Membrane extracts from human fibroblasts were loaded on an EMMPRIN (0.5 mg) affinity column (Carbolink or Aminolink column, Pierce) for 30 min. After washing the column with PBS, the protein was eluted from the affinity column with elution buffer and the samples were analyzed by SDS-PAGE followed by silver staining . A protein of ~55 kDa was identified. These results support the concept that fibroblasts contain a binding protein (receptor) for EMMPRIN that has a mass of ~55 kDa (Figure 6).

We have used the T7Select Phage Display System (Novagen) to identify EMMPRIN binding proteins. We first made a cDNA library using poly(A) + RNA from human fibroblasts known to be sensitive to stimulation by T7Select vector and incubated with T7 packaging extract. Host cells (BLT5403) were then infected with the phage and prepared for biopanning. A solution of purified EMMPRIN (5 mg/ml) was used to coat 24-well plates. After washing with buffer, incubating with blocking solution , and re-washing with water, the wells were incubated with phage lysate and washed again to remove unbound phage. The bound phage were then eluted from the plates with SDS solution, incubated with fresh host cells and lysed for the next round of panning. Five rounds of biopanning were carried out and the final lysate used for plaque assay, PCR amplification and sequencing.

After the five rounds of panning described above, eight clones were obtained. Four of the inserts were of identical size and have been partially sequenced. All four have identical sequences and correspond to a portion of the human interstitial collagenase (MMP-1) sequence. This was an unexpected result but suggests the novel possibility that EMMPRIN binds MMP-1 to the tumor cell surface in similar fashion to the binding of gelatinase A by integrin alphaV-beta3 (27). or TIMP-2:MT1-MMP complex (28).

The presence of MMP-1 at the surface of LX-1 human lung carcinoma cells was confirmed by immunocytochemistry using antibody against MMP-1 (Figure 7).

SUMMARY- In this study we demonstrated that EMMPRIN not only stimulates production of interstitial collagenase (MMP-1), but also forms a complex with MMP-1 at the tumor cell surface. The presentation of MMP-1 complexed to EMMPRIN at the cell surface is important in modifying the tumor cell pericellular matrix to promote cancer invasion. Complex formation was identified using: 1) phage dispay, 2) immunoaffinity chromatography, and 3) immunohistochemistry technology.

(3.c) New Task: Effect of EMMPRIN on production of MMPs by endothelial cells:

In the four years since this grant was written, it has become apparent that tumor angiogenesis plays an important role in the progression of cancer. An obvious question to be addressed is whether the tumor cells produce additional factors besides VEGF that would enhance tumor angiogenesis. Based on our demonstration that EMMPRIN produced by cancer cells stimulates fibroblasts to produce MMPs, we reckoned that EMMPRIN may have a similar effect on endothelial cells.

12/96 to 11/99- To address this question we have incubated EMMPRIN with endothelial cells to determine the effect on MMP production. EMMPRIN purified from stably transfected CHO cells also stimulates production of stromelysin-1, interstitial collagenase, gelatinase A, and TIMP-1 by human umbilical vein endothelial cells cultivated in vitro (Figure displayed in 1998 Report). By comparison, VEGF had a greater stimulatory effect than EMMPRIN on HUVEC synthesis of interstitial collagenase and TIMP-1, but not gelatinase A or stromelysin-1 (29). We concluded that EMMPRIN plays a role in the early phase of tumor angiogenesis by inducing the degradation of endothelial basement membrane. We propose that in vivo, direct contact between circulating tumor cells and endothelial cells lining blood vessels at organs distant from the primary tumor may facilitate tumor cell penetration of the subendothelial basement membrane during metastasis (30). In this scenario, tumor cell EMMPRIN induces endothelial cells to secrete MMPs that subsequently facilitate subendothelial basement membrane degradation; tumor cells then are able to migrate through the rents in the blood vessel wall (30).

Our hypothesis that EMMPRIN is a positive factor in tumor angiogenesis is supported by our recent studies demonstrating more vascularized tumors produced by EMMPRIN-transfected breast cancer cells (see Task 3).

Task 4 (completed) added to original grant application (see 1996 annual report)
Human Keratinocyte EMMPRIN.

12/95 to 11/96- Although initial studies suggested that EMMPRIN is not present in significant amounts on many types of normal adult human cells (2, 3), it subsequently became apparent that EMMPRIN is identical to human basigin and M6 antigen, (12, 13) and is expressed in some physiologically active epithelia during embryonic development, as well as tumor cells. Since keratinocytes have previously been shown to stimulate MMP production by fibroblasts (31) and since epithelial-dermal interactions are important in preserving and repairing skin structures, we sought evidence for the presence of EMMPRIN in keratinocytes. We found that human keratinocytes express EMMPRIN at their cell surface in vivo and in vitro and synthesize EMMPRIN in culture, albeit at a lower level than tumor cells. On characterization of EMMPRIN cDNA obtained from a keratinocyte cDNA library, we found that the deduced amino acid sequence was identical to that of tumor cell EMMPRIN (32). These cDNAs share a common region of 1459 nucleotide residues that differ in only 7 of these residues, only two of which are in the open reading frame and which result in no differences in the amino acid sequence of EMMPRIN. We concluded that human keratinocytes produce EMMPRIN. A role for EMMPRIN at epithelial dermal junctions in tissue repair during wound healing seems highly plausible. Taken together, these data suggest that the function of EMMPRIN is under strict regulation in normal tissues, but this control mechanism may go awry in cancer.

(7) KEY RESEARCH ACCOMPLISHMENTS

- Characterized the EMMPRIN gene
- Demonstrated the prominence of EMMPRIN in human breast cancer
- Demonstrated that EMMPRIN is an important tumorigenic factor
- Identified MMP-1 bound to EMMPRIN on the cell surface
- Demonstrated that EMMPRIN is an enhancing factor for tumor angiogenesis

(8) REPORTABLE OUTCOME

- 7 published manuscripts and 2 manuscripts submitted for publication
- development of monoclonal antibodies to EMMPRIN
- development of an animal model demonstrating EMMPRIN is tumorigenic
- Research grant funded by the NIH to continue work on EMMRIN

(9) CONCLUSIONS

EMMPRIN (TCSF) is a plasma membrane glycoprotein that is present on the surface of breast cancer cells, and is responsible, in part, for the elevated levels of MMPs in peritumoral fibroblasts and endothelial cells. EMMPRIN requires post-translational processing (glycosylation) for its ability to stimulate production of MMPs by target fibroblasts. Transfection of EMMPRIN cDNA into CHO cells resulted in the production of a glycosylated functional protein of similar molecular weight to native EMMPRIN (58 kDa) that was localized to the plasma membrane. EMMPRIN mRNA is expressed in benign and malignant human mammary ducts and acini to a much greater degree than in normal breast ducts. Immunohistochemical studies, however, have indicated that EMMPRIN is also present in normal breast ducts and some other epithelial structures (i.e. macrophages, endothelial cells, keratinocytes). The role of EMMPRIN in these non malignant cells remains to be determined. We have developed IgG monoclonal antibodies to human EMMPRIN which we have employed in immunohistochemistry, immunoassay, and Western blotting.

We have characterized the human EMMPRIN gene. The sum of the exon and intron sizes is 10.8 kb. Transcriptional factor consensus binding sequences have been identified.

We have transfected EMMPRIN cDNA into human MDA-MB-436 breast cancer cells and have demonstrated in 3 independent experiments that these cells are considerable more tumorigenic and invasive than plasmid transfected cancer cells. We have used green fluorescent protein as a convenient marker to examine for metastases in tumor-bearing mice. Intra abdominal metastases were noted with MDA-MB-436/EMMPRIN/GFP transfected cells; MDA-MB-436/GFP transfected cells were slow growing, did not metastasize locally, and did not succumb during the 15 weeks of observation.

Studies were performed to identify a binding site for EMMPRIN on fibroblasts; a putative binding protein of 58 kDa was identified using 2 different experimental approaches. Phage display experiments have demonstrated that EMMPRIN binds MMP-1 to the tumor cell surface in a similar fashion to the binding of progelatinase A to cell surfaces. An inducing effect of EMMPRIN on endothelial cell synthesis of MMPs was identified.

Based on these studies, we propose that inhibition of EMMPRIN function is an important component of the invasive process in breast cancer.

FIGURE LEGENDS

Figure 1A. Schematic illustration of the EMMPRIN/GFP plasmid. A 1.6 kb cDNA representing the entire EMMPRIN sequence was placed at an EcoR 1 site under the control of the CMV promoter in pcDNA 3. GFP cDNA was inserted along with an upstream CMV promoter into the EMMPRIN expression vector between Not I and Xho I sites. A polyadenylation (PA) signal was placed downstream.

Figure 1B. Northern blot analysis of EMMPRIN. ~20 μg of total cellular RNA from plasmid alone-transfected, GFP-transfected, and EMMPRIN/GFP transfected MDA-MB-436 breast cancer cells was size fractionated in a 1% denaturing agarose gel, transferred to a nylon membrane, and incubated with 1.7 kb of ^{32}P-radiolabeled EMMPRIN cDNA as a probe. Blots were analyzed by autoradiography. A single 1.7 kb mRNA transcript corresponding to the known EMMPRIN band was detected at ~20 X greater intensity in EMMPRIN/GFP transfected cells as compared to plasmid alone or GFP transfected cells.

Figure 2A. Identification of green fluorescence in MDA-MB-436 cells. Left panel displays GFP-transfected cells. Middle panel displays EMMPRIN/GFP transfected cells. Right panel displays the expression of EMMPRIN-GFP as a fusion molecule (cDNA controlled by a single CMV promoter) in MDA-MB-436 cells. Fluorescence in the left and middle panels identifies GFP in the cell cytoplasm. GFP fluorescence in right panel identifies the EMMPRIN-GFP fusion protein localized to cell organelles and plasma membranes.

Figure 2B. GFP-transfected tumors are readily visible under fluorescent light. EMMPRIN/GFP transfected MDA-MB-436 breast cancer cells were injected into a NCr nu/nu mouse. Eight weeks later, extensive green colored metastatic tumors (identified with arrows) in the pleura, peritoneum, lymph nodes, liver, and spleen are visible under fluorescent light (left photo). The middle photo demonstrates the same mouse at autopsy illuminated with bright light.

The right photo demonstrates typical tumor size of mice sacrificed at week 15. Tumors from mice injected with EMMPRIN/GFP transfected MDA-MB-436 cells are considerably larger and show prominent blood vessels on the tumor surface as compared to mice injected with GFP-transfected cells. Arrow heads point to tumors.

Figure 3A. MDA-MB-436 breast cancer cells transfected with EMMPRIN/GFP cDNA resulted in enhanced rate of tumor growth after tumor cell implantation into the mammary fat pad of nude mice as compared to GFP-transfected cells. The data represents the mean ± standard error observed in 10 animals in each group. The numbers associated with each symbol refer to the number of mice alive at each time point.

Figure 3B. Comparison of gelatinases produced by MDA-MB-436 cells cultivated in serum-free media and extracts of nude mouse tumors. Spent conditioned media from primary cells cultivated in vitro (left panel) and tumor cell extracts (right panel) were assessed by gelatin substrate zymography. Protein concentration of samples were equalized within each group. Tumor extracts from the EMMPRIN/GFP group had more gelatinolytic activity than GFP-alone (displayed extract from GFP-alone tumor is from the largest tumor in that category).

Figure 4. In situ hybridization of primary tumors from mice injected with EMMPRIN/GFP and GFP-transfected MDA-MB-436 breast cancer cells. Serial sections from tumor tissue (panels 1-4, 9-12) and surrounding non malignant tissue (panels 5-8 and 13-16) were examined. Panels 1 and 9 represent hematoxylin and eosin staining of cancer tissues from EMMPRIN/GFP and GFP tumors, respectively; panels 5 and 13 represent H & E staining of non malignant tissues from EMMPRIN/GFP and GFP tumors, respectively. Cells in the primary tumor mass from mice injected with EMMPRIN/GFP transfected cells revealed widely distributed, specific staining with EMMPRIN, gelatinase A (GLA), and gelatinase B (GLB) antisense riboprobes (panels 2-4). Minimal cell staining was seen in cancer cells from GFP-transfected MDA-MB-436 cells for

EMMPRIN and gelatinase A (panels 10, 11), focal gelatinase B was identified in GFP tumors (panel 12). Non malignant tissues adjacent to the primary tumors from EMMPRIN/GFP mice demonstrated staining for EMMPRIN, gelatinase A, and gelatinase B in mammary ducts (D) and myocytes (M) (panels 6-8). Non malignant tissue from GFP mice revealed no staining for EMMPRIN, gelatinase A, or gelatinase B (panels 14, 15, 16).

Figure 1a

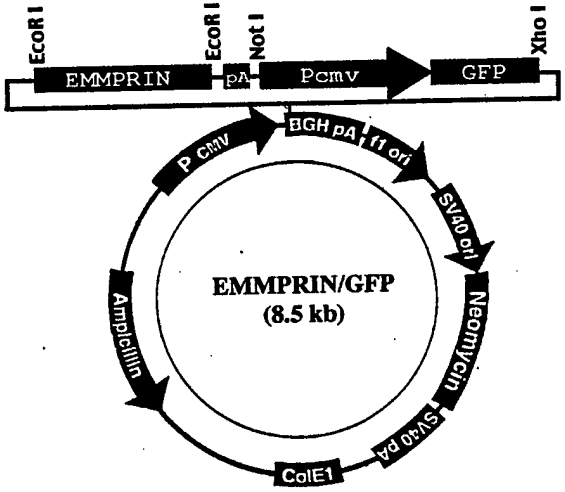


Figure 1b

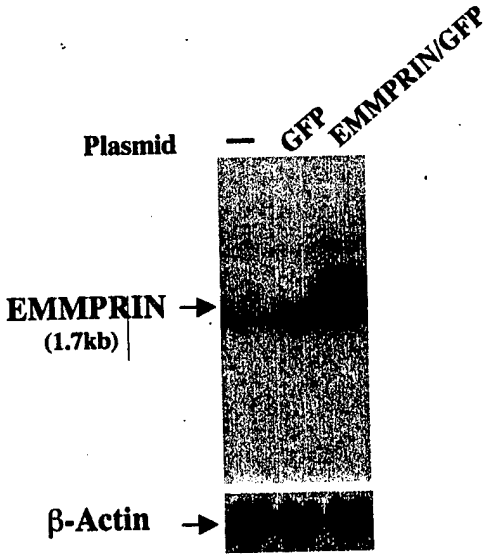
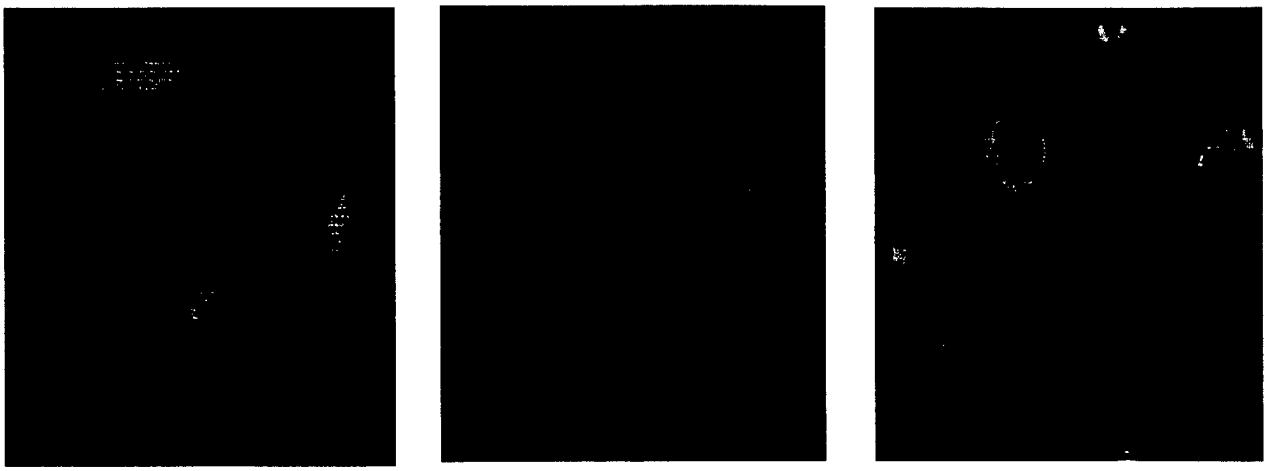


Figure 2

A



B

Figure 3a

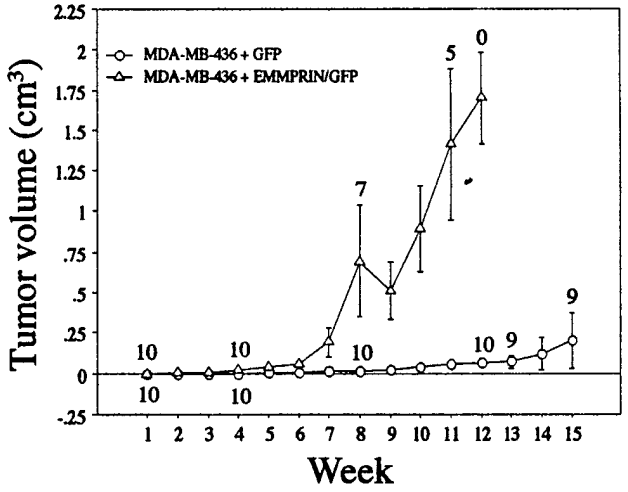


Figure 3b

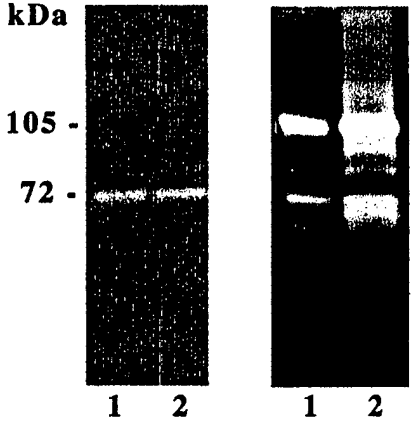
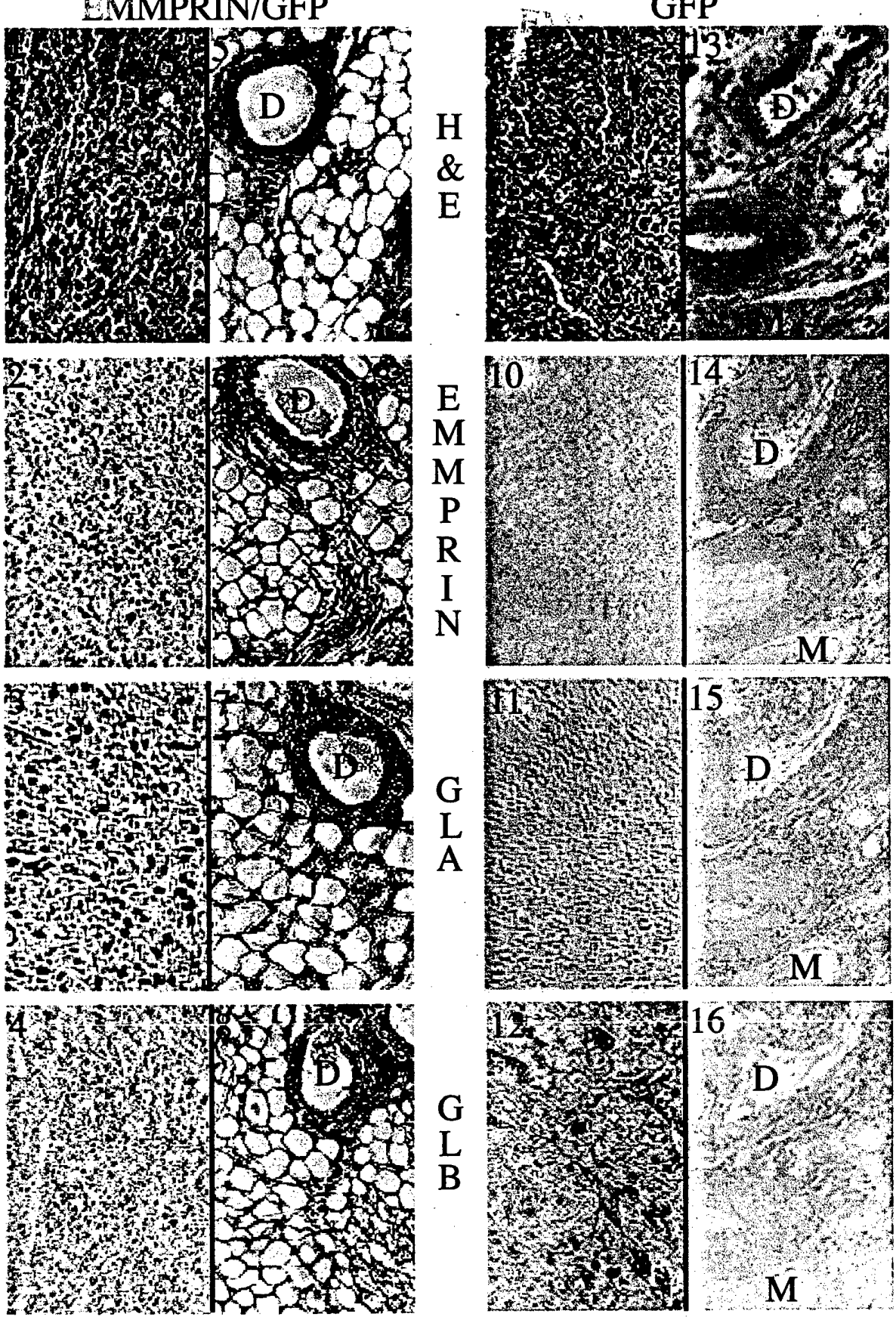


Figure 4


EMMPRIN/GFP
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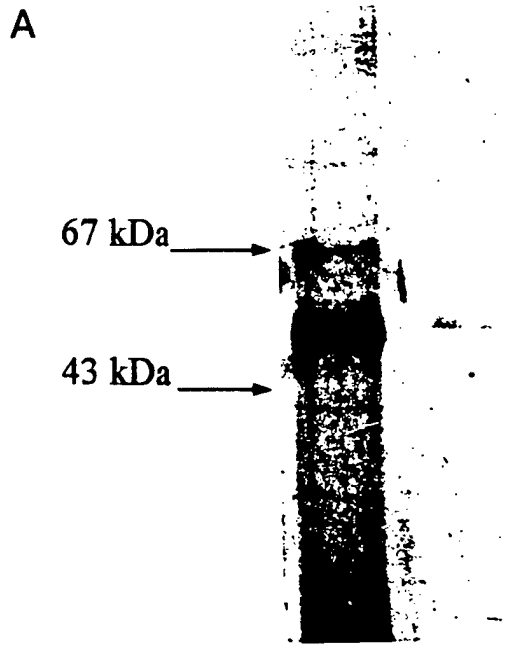

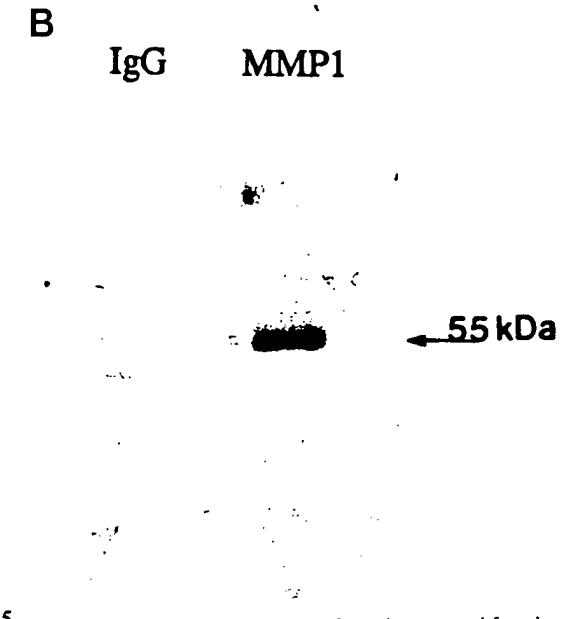

Figure 5 EMMPRIN affinity chromatography of proteins extracted from human fibroblasts. *A*, proteins recovered from chromatography of fibroblast extracts on EMMPRIN-Sepharose were run on SDS-PAGE and silver-stained; two bands (at ~M_r 55,000 and ~M_r 67,000) were detected. *Arrows* indicate positions of the M_r 43,000 and M_r 67,000 markers. *B*, parallel gels to those in *A* were transblotted and reacted with antibody to MMP-1 or secondary antibody only (*IgG*); the ~M_r 55,000 band reacted with anti-MMP-1.

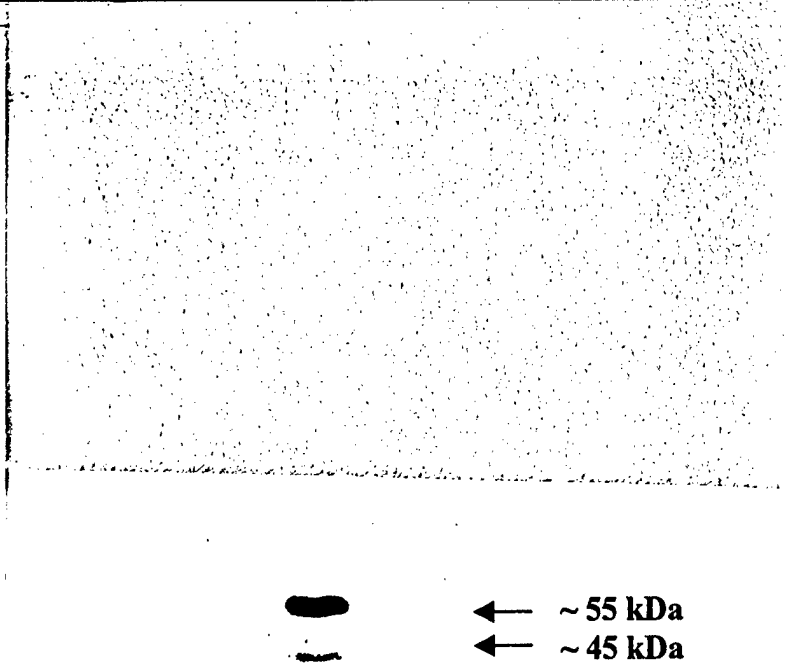

Figure 6 Western blot of immunopurified EMMPRIN with antibody to MMP-1.

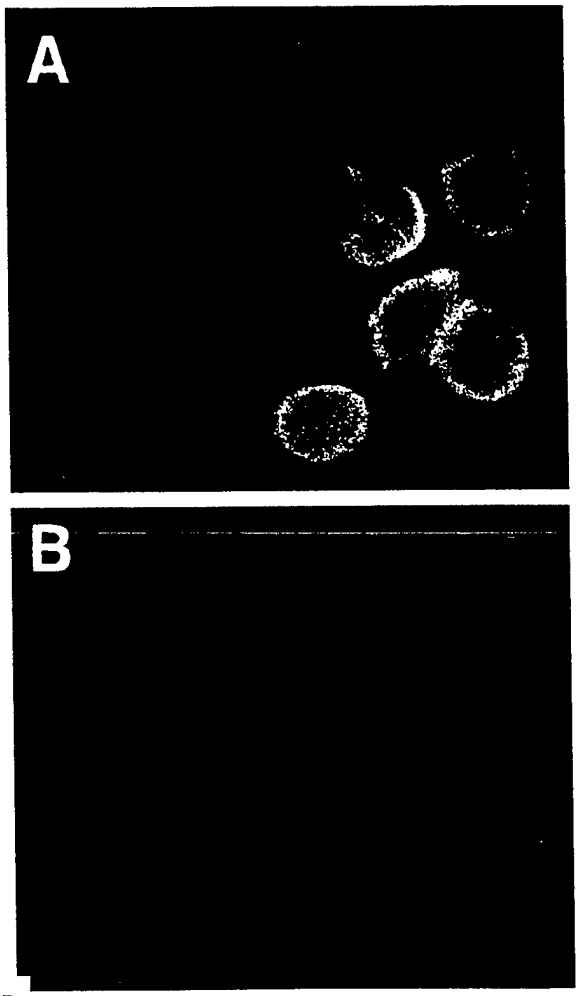

Figure 7 Immunoreactivity of LX-1 human carcinoma cells with antibody to MMP-1. *A*, cells stained with antibody against MMP-1. *B*, cells stained with secondary antibody only.

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(12) LIST OF PERSONNEL RECEIVING PAY FROM THIS EFFORT
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(13) APPENDICES: Reprints of Publications 1-5, 7-10.

[CANCER RESEARCH 55, 434–439, January 15, 1995]

The Human Tumor Cell-derived Collagenase Stimulatory Factor (Renamed EMMPRIN) Is a Member of the Immunoglobulin Superfamily[1]

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ABSTRACT

Tumor cell-derived collagenase stimulatory factor, renamed extracellular matrix metalloproteinase inducer (EMMPRIN), is a M_r ~58,000 glycoprotein which is located on the outer surface of human tumor cells and which interacts with fibroblasts to stimulate expression of several matrix metalloproteinases in the fibroblasts. In this study, we have used several approaches to isolate a complementary DNA encoding EMMPRIN. Several peptide sequences obtained from the isolated M_r 58,000 glycoprotein are found in the translated complementary DNA clone, verifying its identity. Computer database searches indicate that EMMPRIN is a member of the immunoglobulin superfamily and that the deduced amino acid sequence of EMMPRIN is identical to that recently reported for human basigin and M6 antigen, molecules of previously undetermined biological function.

INTRODUCTION

Degradation of extracellular matrix components of the basement membrane and interstitial matrix by MMPs[6] is a crucial step in tumor cell invasion and metastasis (1–3). The role of tumor cell-fibroblast interactions in regulation of MMP levels in neoplasms has been demonstrated by several investigators, including ourselves (4–7). The recent finding (8–11) that, *in vivo*, some tumor-associated MMPs are mainly synthesized in peritumoral fibroblasts, rather than in tumor cells themselves, is consistent with a major role for these interactions in tumorigenesis *in vivo*.

We have shown that tumor cells in culture stimulate fibroblasts to produce high levels of collagenase and that a factor (previously termed TCSF) that is associated with tumor cell membranes, but also released into medium conditioned by tumor cells, is responsible for this stimulation (6, 12, 13). We have immunoaffinity purified the M_r ~58,000 TCSF from a human lung carcinoma cell line, LX-1 (13, 14), and demonstrated that addition of this purified factor to cultured fibroblasts stimulates expression, not only of interstitial collagenase (MMP-1), but also of fibroblast-derived stromelysin-1 (MMP-3) and M_r 72,000 gelatinase (MMP-2) (15, 16). While immunohistochemical studies have shown that TCSF is highly enriched around the outer surface of tumor cells and absent from most normal cells *in vivo* (17), recent studies from our laboratory[7] have shown that TCSF is also present on the surface of normal human keratinocytes, where it presumably plays a role in regulating stromal MMPs (18). For these reasons, we have now renamed this factor EMMPRIN to indicate its role in extracellular matrix metalloproteinase induction via normal, as well as pathological, cellular interactions.

To help understand the chemical and biological nature of EMMPRIN and its relationship to other proteins, we have attempted to isolate cDNA clones for the protein. Oligonucleotide primers derived from peptide sequences were used to isolate EMMPRIN cDNAs by RT-PCR. Analysis of the cDNA-derived amino acid sequence of EMMPRIN indicates that it is a member of the immunoglobulin superfamily. Interestingly, the sequence is identical to two recently reported human cDNAs of unknown function, *i.e.*, human basigin (19) and M6 antigen (20). Thus, our studies provide one important function for these proteins, namely intercellular stimulation of MMP synthesis.

MATERIALS AND METHODS

Amino Acid Sequencing. Previously, we have reported the amino acid sequences for the NH_2-terminus of EMMPRIN and four peptides derived from EMMPRIN after trypsin digestion (14). We have now sequenced two more peptides derived from EMMPRIN in the same manner as described previously (14). Briefly, immunopurified EMMPRIN was subjected to SDS-PAGE and blotted to a nitrocellulose membrane. The EMMPRIN band was revealed by staining with Ponceau S. After destaining, the protein band was cut from the membrane and digested with trypsin at a ratio of 1:20 (w/w). The peptides were separated by reverse phase HPLC, and the samples were subjected to automated Edman degradation.

cDNA Synthesis. RNA was prepared from LX-1 cells by a routine procedure using guanidinium thiocyanate (21), and poly(A)$^+$RNA was isolated using the Mini Ribosep mRNA isolation kit according to the manufacturer's instructions (Collaborative Biomedical Products, Bedford, MA). First-strand cDNA was synthesized by reverse transcription of 1 μg of poly(A)$^+$ RNA using Moloney murine leukemia virus reverse transcriptase, according to the manufacturer's instructions (GIBCO-BRL, Gaithersburg, MD) in the presence of random hexamers or specific primers, depending on the desired reaction product. The resulting reaction mixture was digested with RNase H and used as a template for PCR.

DNA Amplification. PCR-amplified DNA fragments were generated with a Perkin-Elmer Cetus DNA thermal cycler (Norwalk, CT) using a gene amplification kit (Perkin Elmer) according to the manufacturer's instructions. Briefly, 100 μl of reaction mixture contained 100 ng of cDNA pool, 10 μl of 10X PCR buffer (provided in the kit), 16 μl of each deoxynucleotide triphosphate at 1.25 mM, 5 μl each of 20 μM primers, and 0.5 μl of Taq-DNA polymerase (2.5 units per assay). Samples were subjected to 30 cycles at the following conditions: 1 min at 94°C for denaturation; 1 min at 48°C for annealing; and 1.5 min at 72°C for elongation. A final elongation step consisted of a 72°C incubation for 10 min. Amplified products were separated by agarose gel electrophoresis and were identified by ethidium bromide staining. Where reamplification of a PCR product was necessary, samples were applied to low-melting point agarose gels (FMC, Rockland, ME); bands were excised, melted, and purified on Spin Bind extraction cartridges (FMC).

Received 8/26/94; accepted 11/9/94.

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[1] This work was supported by Grant CA 38817 (to C. B.) from the NIH.

[2] This article is dedicated to the memory of Dr. Chitra Biswas, who passed away August 26, 1993. Requests for reprints should be addressed to Dr. Bryan Toole at Department of Anatomy and Cellular Biology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111.

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[6] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; PCR, polymerase chain reaction, RT-PCR, reverse transcriptase-PCR; TCSF, tumor cell-derived collagenase stimulatory factor; bp, base pair(s); kb, kilobase.

[7] R. DeCastro, Y. Zhang, H. Guo, H. Kataoka, and C. Biswas. Human keratinocytes express EMMPRIN, an extracellular matrix metalloproteinase inducer, manuscript in preparation.

Generation of an Authentic cDNA for EMMPRIN Peptide #51. Two 17-mer, degenerate, oligonucleotide mixtures, based on the previously obtained amino acid sequence of EMMPRIN peptide #51 (14), were synthesized. One of these, termed A, was synthesized in the sense direction corresponding to amino acids 2–7; the other, termed C′, was synthesized in the antisense direction corresponding to amino acids 13–18. The sequences of the oligonucleotide mixtures are shown in Table 1. These mixtures were used as primers in RT-PCR to generate an authentic EMMPRIN cDNA corresponding to amino acid residues 2–18 of peptide #51, using LX-1 cell mRNA as the initial template. The primers had 8-bp adapters with restriction site sequences (*Eco*RI for the 5′-end of A and *Pst*I for the 5′-end of C′; not shown in Table 1), allowing subsequent cloning of the product in the event that difficulty was encountered in directly ligating the product into the pCRII vector. The resulting PCR products were sized by electrophoresis against known DNA markers in 6% agarose (NuSieve GTG low melting point agarose; FMC). Several products of different intensities were identified, including the expected 66-bp product corresponding to #51 (50-bp fragment plus 2 × 8 bp adapters at 5′ and 3′ ends). The band corresponding to this size was cut out from the agarose gel and reamplified using the same primers A and C′ as described above. After checking the size of the reamplified product, all of which was 66 bp, the reaction mixture was used directly for ligating into the pCRII vector system (Invitrogen). Recombinants were selected as white colonies on plates containing 5-bromo-4-chloro-3-indolyl-*b*-D-galactoside. Plasmid DNA was isolated from seven of these colonies, and the sizes of the inserts were analyzed by PCR amplification using M13 primers (forward and reverse), followed by agarose gel electrophoresis.

All seven recombinants had inserts of the expected sizes. DNA from two of these clones was sequenced by the dideoxy-mediated chain termination method (22) using a double-stranded DNA cycle sequencing kit (GIBCO-BRL). The nucleotide sequence of the inserts from both clones exactly matched the amino acid sequence of peptide #51 (Fig. 1). However, the nucleotide sequence varied at three positions, all within the primer sequences, as shown in *bold* in Fig. 1. This suggests that several of the degenerate primers were used by the template cDNA during amplification. The use of degenerate oligonucleotide mixtures as primers often leads to variations of this kind in the regions of the cDNA that correspond to the primers (23). To avoid these regions of variation, we designed primers (B/B′) based on the sequence of the central part of the cDNA (Fig. 1) for use in the overlap extension reactions described below.

Generation of cDNAs Corresponding to the 5′ and 3′ Regions of EMMPRIN. To obtain a cDNA that includes the region of EMMPRIN that is 5′ to peptide #51, primer B′ (designed as described above) was used in the PCR in combination with primer D, a degenerate mixture corresponding to part of the previously sequenced NH_2-terminal peptide #59, derived from EMMPRIN (Ref. 14, see Table 1). The PCR products were directly ligated into pCRII (Invitrogen) and used to transform *Escherichia coli*. Insert-containing white colonies were selected, and the plasmid DNAs were isolated, sized, and sequenced. One of these cDNAs, TALT5j, was used for further study.

To obtain a cDNA that includes the region of EMMPRIN that is 3′ to peptide #51, we used the rapid amplification of cDNA ends protocol as described previously (24). A pool of cDNA was prepared from poly(A)$^+$ RNA of LX-1 cells using the dT_{17} adapter primer E of 5′-GAATTCGAATTC-GATATCTTTTTTTTTTTTTTTT. The reaction mixture was then amplified in PCR using the dT_{17} adapter primer E and primer B, derived from peptide #51 (Table 1). After ligating the PCR products into the pCRII vector, transforming *E. coli*, and selecting white colonies, the plasmid DNA was isolated, and the inserts were sized and sequenced. One of these cDNAs, TALT3g, was used for further study.

Table 1 *EMMPRIN peptide sequences and derived oligonucleotide primers*

The amino acid sequences of the peptides for which corresponding oligonucleotide primers were synthesized are underlined. Oligonucleotide sequences A, B, and D are shown in the sense orientation, whereas B′ and C′ are in the antisense orientation. Primers A, C′, and D are degenerate mixtures; primers B and B′ are specific sequences derived as described in the text and Fig. 1. The nucleotide sequence, GARGA, highlighted in bold print at the 3′ end of primer D fortuitously matches a portion of the 5′ untranslated region of EMMPRIN (with the sequence, GAGGA) and thus amplified a cDNA beginning at this position (see Fig. 2).

Peptide sequence		Oligonucleotide primer[a]
#51 SELHIENLNMEADPGQYR	A:	5′-GAR-YTN-CAY-ATM-GAR-AA-3′
SELHIENLNMEADPGQYR	B:	5′-AAC-CTG-AAC-ATG-GAG-GCC-GA-3′
SELHIENLNMEADPGQYR	B′:	5′-TC-GGC-CTC-CAT-GTT-CAG-GTT-3′
SELHIENLNMEADPGOYR	C′:	5′-CZ-RTA-YTG-NCC-NGG-RTC-3′
#59 AAGTVFTTVEDLGSK	D:	5′-TTY-ACN-ACN-GTN-GAR-GA-3′

[a] M = A, C, or T; N = A, C, G, or T; Y = T or C; R = A or G; Z = G or T.

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-------Primer A------>                          <-----Primer C'-------

GAA-CTT-CAC-ATT-GAG-AAC-CTG-AAC-ATG-GAG-GCC-GAT-CCC-GGC-CAA-TAC-CG

-E---L---H---I---E---N---L---N---M---E---A---D---P---G---Q---Y---R-
```

Fig. 1. Sequence of authentic cDNA for EMMPRIN peptide #51. The positions of primers A and C′ used to obtain the authentic cDNA for peptide #51 are shown on the *top line*; the nucleotide sequence of one of the cDNAs obtained from PCR is given on the *second line*; the amino acid sequence of peptide #51 (residues #2–18) is on the *third line*. The amino acid sequence deduced from the nucleotide sequence is identical to that obtained by amino acid sequencing. A second clone was sequenced, and differences were found in the positions highlighted in *bold print* at the third, sixth and forty-fifth positions of the nucleotide sequence; all of these lie within regions corresponding to the primers, and this variation was presumably due to the use of degenerate oligonucleotides as primers. Primers B (*underlined*) and B′ were designed from the central part of this cDNA.

Construction of a cDNA Encoding the Complete EMMPRIN Sequence. The inserts of the 5′ and 3′ cDNA clones for EMMPRIN (TALT5j and TALT3g) overlapped by 20 nucleotides and thus were used in the PCR-based, overlap extension technique (25) to yield a single cDNA with the complete open reading frame for EMMPRIN.

First, the cDNAs corresponding to the 5′ region (TALT5j) and the 3′ region (TALT3g) were amplified by PCR in two separate reactions. For TALT5j, a 30-mer sense primer, composed of a 13-bp *Bam*HI adapter at the 5′ end followed by a 17-mer corresponding to the 5′ terminus of TALT5j, was used (primer F, 5′-CGCGGATCCCGGCGAGGAATAGGAATCATG); the antisense primer was a 20-mer which corresponded to the 3′ terminus of TALT5j (primer B′ in Table 1). For TALT3g, a 20-mer sense primer corresponding to the 5′ terminus of TALT3g (primer B in Table 1) and the dT_{17} adapter primer E (5′-GAATTCGAATTCGATATCTTTTTTTTTTTTTTTT) were used. The amplified products were electrophoresed in agarose, and the bands corresponding to 0.6 kb (TALT5j) or 1.1 kb (TALT3g) were identified by ethidium bromide staining and excised.

In the third and final reaction, the gel-purified products from both the above reactions were used as templates for fusion by overlap extension (25). For this reaction, the 30-mer primer F (5′-most primer), used above for amplification of TALT5j, was used with the dT_{17} adapter primer E (3′-most primer). The 1.6-kb DNA product that was generated in this PCR was ethanol precipitated, agarose gel-purified, and subcloned into pBluescript. Sequencing of this cDNA confirmed its identity with the combined sequence of the two separate cDNAs. The complete sequence is shown in Fig. 2.

Northern Blot Analysis. Northern blot analysis was performed as described previously (16). Ten μg of total RNA was electrophoresed on a 1% agarose gel and transferred to nitrocellulose membrane. The blot was then hybridized overnight with EMMPRIN cDNA, which had been radiolabeled by nick-translation with ^{32}P-labeled dCTP. After washing, the filter was exposed to Kodak XR-5 film at −80°C for 48 h.

Deletion Analyses. The strategy used for making deletion constructs was adapted from that used to create the full-length EMMPRIN cDNA, *i.e.*, overlap extension (25). The primers used for the following reactions were primer F, 5′-CGCGGATCCCGGCGAGGAATAGGAATCATG-3′; primer G, 5′-ACG-GAGCCTCCGGGGTGAAGGCTGTGAAGTCG-3′; primer H, 5′-ACGGG-CCTCCCAGAAGCCACCTGGCCGCCCTC-3′; primer I, 5′-GCGTGCGC-AGCCACGAGAAGCGCCGGAAGCCC-3′; and primer J, 5′-TTCATCT-ACTAGTAGCGCCGGAA-3′.

The extracellular immunoglobulin domain I deletion construct was made as follows: (*a*) two PCRs, using EMMPRIN cDNA as template, were used to synthesize DNA fragments on each side of the desired deletion, one with the primers F (a *Bam*HI adapter plus the 5′ end of the EMMPRIN cDNA sequence) and reverse complement of G, and the other with primer G and the dT_{17} adapter primer E. Primer G is composed of sequences from each side of the desired deletion, *i.e.*, nucleotides 50–63 linked directly to 319–336 (Fig. 2). PCR products were analyzed and purified on 1% low-melting point agarose. DNA of expected sizes, 108 bp from the first reaction and 1306 bp from the second reaction, were cut from the gels and incubated in 300 μl H_2O at

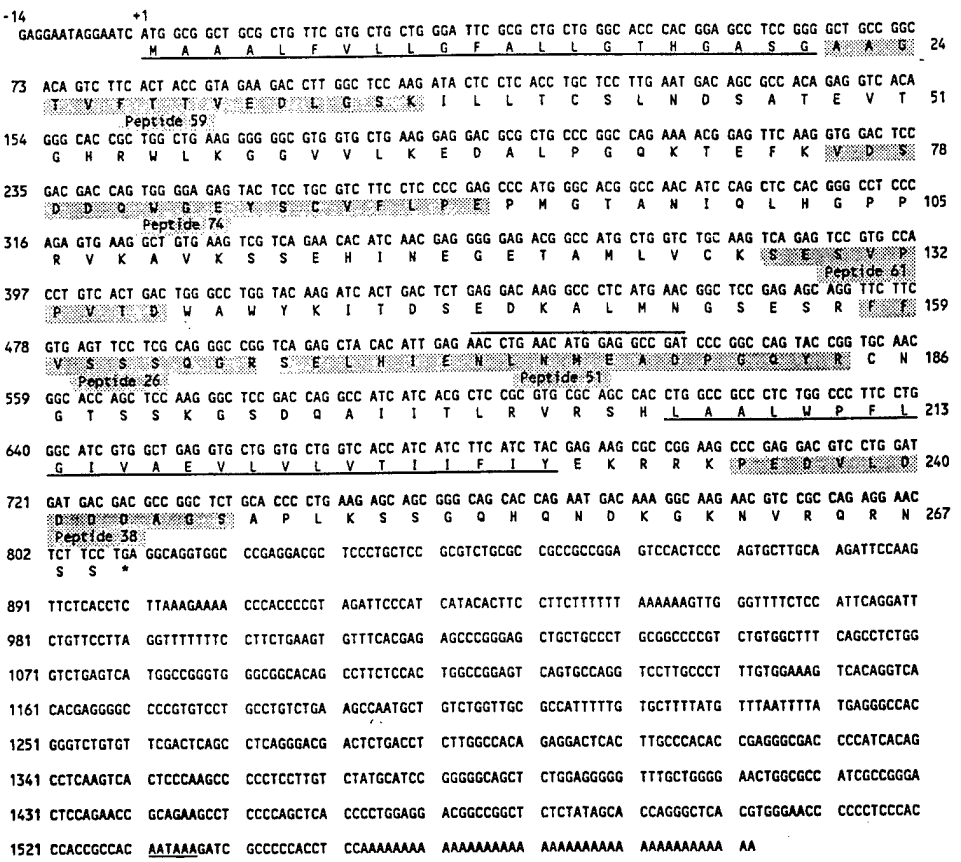

Fig. 2. Sequence of human EMMPRIN cDNA.[8] Nucleotide sequence and derived amino acid sequence for human EMMPRIN; nucleotides are numbered on the *left* and amino acids on the *right*. The putative signal and transmembrane amino acid sequences and the polyadenylation signal are *underlined*. The *shaded regions* indicate the deduced amino acid sequences that match the six peptides derived from EMMPRIN. The 20-nucleotide overlapping region of the 5′ and 3′ cDNAs, TALT5j and TALT3g, is indicated by a *line* above nucleotide positions #517–536. The stop codon is marked by an *asterisk*.

65°C for 10 min; (*b*) a PCR was performed with 10 ng each of these two fragments, plus primer F and the dT_{17} adapter. The PCR product was precipitated with 0.3 M ammonium acetate and 2 volumes of ethanol, *Eco*RI, and electrophoresed on 1% low-melting point agarose. DNA with the expected size, 1382 bp, was cut from the gel and purified on Spin bind extraction cartridge; and (*c*) the purified DNA was ligated into pBluescript, and used to transform XL-1 blue cells. Plasmid DNA was isolated from positive (white colony) transformants and confirmed by sequencing.

To create constructs with deletions in the extracellular immunoglobulin domain II, the transmembrane domain, and the cytoplasmic domain, the same approach was used with appropriate primer pairs. For deleting the extracellular immunoglobulin domain II, primers F and the reverse complement of H, and primer H and the dT_{17} adapter, were used to make fragments on each side of the desired deletion. Primer H contains sequences from both sides of the region we wished to delete, bases 305–318 linked directly to 610–627 (Fig. 2). To delete the transmembrane domain, the two fragments were made with primers F and the reverse complement of I, and primer I with the dT_{17} adaptor. Primer I consists of bases 602–615 linked to 688–705 (Fig. 2), sequences on each side of the desired deletion. Primer F and the dT_{17} adapter were used in the final PCR to make the fused deletion product from the two fragments. To delete the cytoplasmic domain, two premature stop codons were inserted into the EMMPRIN cDNA. PCR fragments were made from template cDNA using primers F and the reverse complement of J, and J with the dT_{17} adapter. Primer J corresponds to nucleotides 679–701 with T residues substituted for G and A at positions 688 and 691, respectively (Fig. 2) to create new termination codons. The two fragments were fused by overlap extension with primer F and the dT_{17} adapter. These deletion constructs were also subcloned into pBluescript and used to transform XL-1 blue cells.

To express each deletion construct, 20 μl of overnight cultures of each bacterial stock were added to 2 ml of Luria broth containing 0.2% glucose and 50 μg/ml ampicillin. After incubating at 37°C for 2 h, the cells were further incubated in the absence or presence of 0.5 mM isopropylthio-*b*-D-galactoside at 37°C for 3 h. The cells were collected by centrifugation and extracted by 30 μl of 2 × SDS-PAGE sample buffer. The cell extracts were separated on 15% SDS-PAGE and then analyzed for cross-reactivity with the monoclonal antibody E11F4 by Western blotting (26).

RESULTS

Amino Acid Sequences of Peptides Derived from EMMPRIN. Previously, we have reported the amino acid sequence of four peptides termed #26, #51, #59, and #61, obtained by HPLC fractionation of tryptic peptide digests of EMMPRIN (14). Subsequently, we have sequenced two additional peptides, #38 and #74, from EMMPRIN. The sequences of the six peptides are shown in Table 2.

Isolation of EMMPRIN cDNAs. Several attempts to obtain EMMPRIN cDNA clones by screening an LX-1 library with degenerate or best-guess oligonucleotide probes, derived from the peptide sequences, were unsuccessful. Therefore, the following steps were taken to obtain a cDNA for EMMPRIN. First, a small EMMPRIN-specific cDNA corresponding to a single peptide-derived sequence, peptide #51, was generated by RT-PCR with degenerate primers. Isolation of this cDNA confirmed the presence of an mRNA contain-

Table 2 *Amino acid sequences of peptides derived from EMMPRIN*

Peptides #38 and #74 (marked with an asterisk) are reported here for the first time; peptides #26, #51, #59, and #61 were reported previously (14).

Peptide #26:	Phe-Phe-Val-Ser-Ser-Ser-Gln-Gly-Arg
Peptide #38*:	Pro-Glu-Asp-Val-Leu-Asp-Asp-Asp-Asp-Ala-Gly-Ser-
Peptide #51:	Ser-Glu-Leu-His-Ile-Glu-Asn-Leu-Asn-Met-Glu-Ala-Asp-Pro-Gly-Gln-Tyr-Arg
Peptide #59:	Ala-Ala-Gly-Thr-Val-Phe-Thr-Thr-Val-Glu-Asp-Leu-Gly-Ser-Lys
Peptide #61:	Ser-Glu-Ser-Val-Pro-Pro-Val-Thr-Asp
Peptide #74*:	Val-Asp-Ser-Asp-Asp-Gln-Trp-Gly-Glu-Tyr-Ser-X-Val-Phe-Leu-Pro-Glu-

[8] The sequence reported in this publication has been deposited in the GenBank data base (accession no. L10240).

ing the EMMPRIN-derived sequence in LX-1 cells and provided us with a correct cDNA probe to be used for generation of larger EMMPRIN cDNAs.

After sequencing the small cDNA corresponding to peptide #51, a unique reverse complement primer derived from it was used in RT-PCR, together with a degenerate primer made from the amino terminal peptide #59, to obtain a longer cDNA. Sequencing of one of the isolated cDNAs (TALT5j; insert size, 0.6 kb) revealed the following characteristics: (*a*) as expected, the sequences at the 5′ and 3′ ends corresponded to the two primers, D and B′, which were based on portions of peptides #59 and #51, respectively; and (*b*) the complete sequences encoding peptides #26, #61, and #74 (Table 2) were present within the cDNA, demonstrating that the cDNA encodes an authentic EMMPRIN sequence.

An unexpected result from the above approach was that although the sequence of primer D was present at the 5′ end of the cDNA, the complete sequence corresponding to peptide #59, from which D is derived, was found to begin 72 nucleotide residues downstream from the primer sequence. Thus, it appears that primer D annealed with a region of the 5′ untranslated sequence of EMMPRIN and amplified a cDNA that begins in the 5′ untranslated region. A possible explanation of this event comes from comparison of the sequence of this cDNA with sequences recently obtained from an EMMPRIN cDNA derived from a human keratinocyte cDNA library.[7] The sequence at the 3′ end of primer D (Table 1) corresponds exactly with a sequence within the 5′ untranslated region, namely GAGGA, for the keratinocyte-derived as well as LX-1-derived EMMPRIN cDNAs. Therefore, this unexpected circumstance led to additional information about the 5′ untranslated end of our cDNA.

Thus, in summary, the 5′ cDNA (TALT5j) corresponds to nucleotide residues −14 to 536, defining +1 as A in the ATG start codon (Fig. 2). It begins with a portion of 5′ untranslated sequence, then contains a methionyl initiation codon at the start of a sequence encoding a region with the properties of a signal peptide (amino acid residues #1–21 in Fig. 2). This is followed by the sequence encoding peptide #59 (residues #22–36), corresponding to the NH_2-terminus of the mature protein. The cDNA continues through to the 3′ end primer, B′, encoding part of the amino acid sequence corresponding to peptide #51 (residues #173–178).

The rapid amplification of cDNA ends technique (24) was applied to obtain the 3′ cDNA, once more using a primer based on part of the authentic nucleotide sequence from the peptide #51 cDNA, together with a universal 3′ oligo dT_{17} adapter primer. Sequence analysis of the PCR products indicated that one of the inserts (TALT3g), 1.1 kb in size, begins at the 5′-terminus with the sequence corresponding to primer B, continues with the sequence of the COOH-terminus of peptide #51 (Table 1), and includes a perfect match with the amino acid sequence of peptide #38 (Table 2). The sequence of TALT3g corresponds to nucleotides 517–1592 in Fig. 2. This sequence also contains a stop codon at nucleotide positions #808–810 and a 3′ untranslated region containing a poly-adenylation signal and a poly(A) tail (Fig. 2).

Finally, the 5′ and 3′ cDNAs generated above were fused by the overlap extension method (25) to yield a single 1.6-kb cDNA. The full sequence of this cDNA, its deduced translation product, and the tryptic peptide sequences are shown in Fig. 2.

Northern Blot of mRNA for EMMPRIN. To ascertain the size of mRNA for EMMPRIN, total RNA derived from LX-1 cells was analyzed by Northern blotting using the 5′ cDNA (TALT5j) as a probe. A single band of ~1.7 kb was observed (Fig. 3). This mRNA size is close to the size of cDNA obtained after fusion of the TALT5j and TALT3g cDNAs, *i.e.*, 1.6 kb.

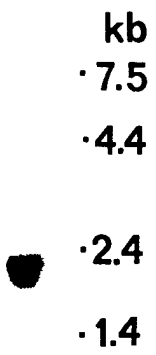

Fig. 3. Northern blot of LX-1 RNA probed with EMMPRIN cDNA. Ten μg of total RNA was loaded and probed with nick-translated, radiolabeled cDNA (TALT5j). Total radioactivity used was 1×10^6 cpm/ml, and the duration of film exposure was 18 h.

Analysis of EMMPRIN cDNA Sequences. The Northern blots indicate that the EMMPRIN composite cDNA corresponds to all but about 100 nucleotides of the mRNA. Since the clone contains a poly(A) tail, this means that the 100 bases are probably located at the 5′-end of the 5′ untranslated region, making the total untranslated region about 115 nucleotides in length.

The cDNA encodes a 269-amino acid residue polypeptide that contains a putative signal peptide of 21 amino acid residues, an extracellular domain of 185 amino acid residues, a putative transmembrane region (residues 206–229), and a carboxy-terminal cytoplasmic domain of 39 amino acid residues (230–269). The transmembrane region includes three leucines (residues 206, 213, and 220) and a phenylalanine (residue 227), occurring every seventh residue, a characteristic feature of the leucine zipper motif (Fig. 2). The extracellular region contains four cysteinyl residues spaced in a manner that gives rise to two distinct domains with the characteristics of proteins in the immunoglobulin superfamily. These residues in EMMPRIN are located at amino acid residue positions 41, 87, 126, and 185.

Expression of Recombinant EMMPRIN Protein and Deletion Constructs. Although the EMMPRIN cDNA encodes amino acid sequences identical to those of peptides directly isolated from immunoaffinity-purified EMMPRIN protein, we reconfirmed the identity of the EMMPRIN cDNA by showing that the recombinant protein is recognized by activity-blocking monoclonal antibody. To accomplish this, the recombinant protein was expressed in pBluescript and then assayed by Western blotting with EIIF4, a monoclonal antibody that blocks the activity of EMMPRIN protein from LX-1 cells (13, 14). As shown in Fig. 4*B*, *Lane 2*, EIIF4 reacts with the recombinant EMMPRIN protein. Two immunoreactive bands were obtained; these correspond in size to the two forms of EMMPRIN noted previously (14) and most likely arise by proteolysis.

The bacterial recombinant protein was also used to determine the approximate location of the epitope for E11F4, taking advantage of the lack of posttranslational modifications that would interfere with such studies on the native, immunoaffinity-purified protein. Modified EMMPRIN expression plasmids were made containing deletions in four locations. As seen in Fig. 4*A*, these deletions were: (*a*) deletion of the extracellular immunoglobulin domain I (ΔECI); (*b*) deletion of the extracellular immunoglobulin domain II (ΔECII); (*c*) deletion of the transmembrane domain (ΔTM); and (*d*) deletion of the cytoplasmic domain (ΔCYT). XL-1 blue cells were transformed with the deletion expression pBluescript plasmids, and the expressed proteins were analyzed by SDS-PAGE and Western blotting with the monoclonal antibody E11F4. As seen in Fig. 4*B*, all the plasmids produce protein that is immunoreactive, except for the plasmid lacking immunoglobulin domain I.

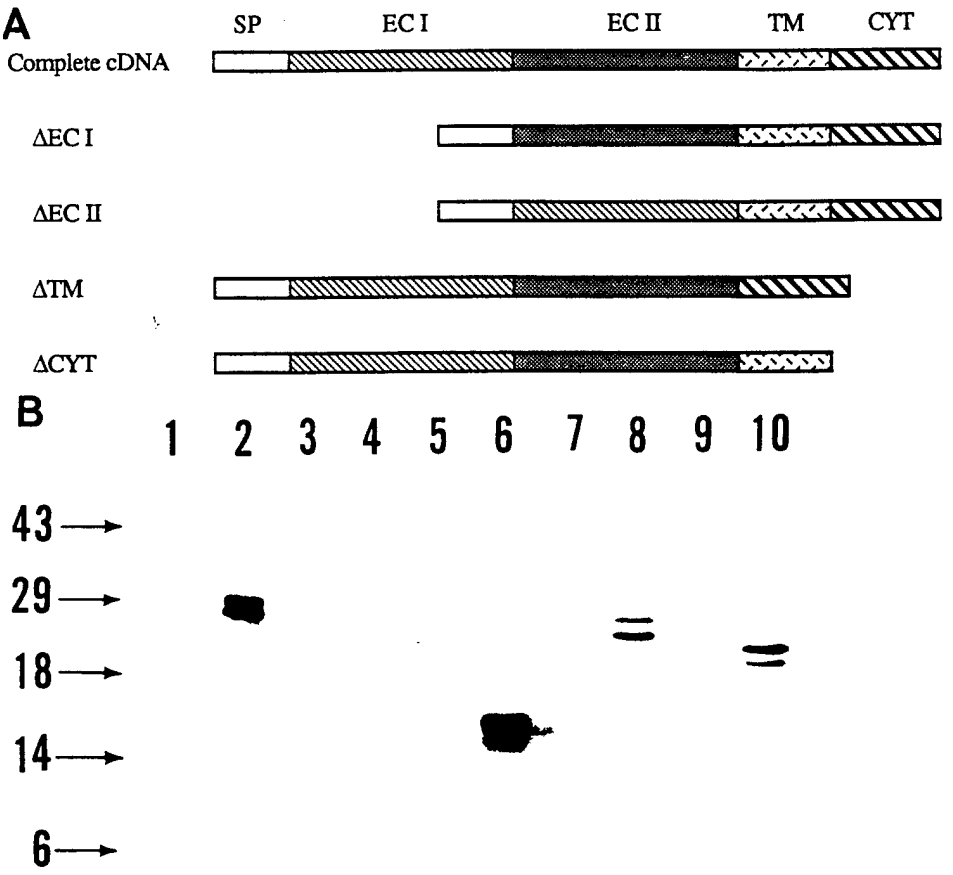

Fig. 4. Localization of EIIF4 epitope in recombinant EMMPRIN. *A*, strategy for deleting the four major domains of the EMMPRIN cDNA. ΔECI and ΔECII, deletion of one of two immunoglobulin-like domains within the extracellular region; ΔTM, deletion of transmembrane domain; ΔCYT, deletion of cytoplasmic domain; *SP*, signal peptide; *B*, Western blot. The transformed XL-1 blue cells were incubated in the absence (*Lanes 1, 3, 5, 7,* and *9*) or presence (*Lanes 2, 4, 6, 8,* and *10*) of 0.5 mM isopropyl-β-D-thiogalactopyranoside and processed for Western blotting. *Lanes 1* and *2*, recombinant EMMPRIN without deletion; *Lanes 3* and *4*, ΔECI; *Lanes 5* and *6*, ΔECII; *Lanes 7* and *8*, ΔTM; *Lanes 9* and *10*, ΔCYT. Molecular weight marker positions in kilodaltons are shown.

These results demonstrate that our cDNA encodes the protein which is reactive with our activity-blocking monoclonal antibody and that the antibody epitope exists in the extracellular immunoglobulin domain I. This, in turn, implies that the functional site of the metalloproteinase stimulatory activity of EMMPRIN is likely to be localized to sequences contained in the immunoglobulin domain I region.

DISCUSSION

We have isolated and fused two overlapping cDNA clones, using the polymerase chain reaction, which together encode the complete, 269-amino acid open reading frame for EMMPRIN. The identity of the clones was confirmed by comparison to several peptide sequences derived from immunoaffinity-purified EMMPRIN. Recognition of the translation product by the activity-blocking monoclonal antibody EIIF4 further confirms the identity of the cDNAs as the desired EMMPRIN clones. In addition, we have recently isolated an EMMPRIN cDNA from a human keratinocyte cDNA library. The open reading frame of the latter cDNA has an identical sequence to the cDNA obtained in the present study by PCR-based techniques, except for two nucleotide residues; however, the deduced amino acid sequences are identical for the two cDNAs.[7] Finally, we have recently demonstrated that recombinant EMMPRIN isolated from CHO cells transfected with EMMPRIN cDNA stimulates metalloproteinase production in fibroblasts.[9]

The composite cDNA obtained in the present study has a small 5′ untranslated region, followed by an initiation codon and sequences that have the properties of a signal peptide sequence, when using the rules of von Heijne (27). The subsequent codons agree perfectly with our amino terminal peptide sequence for the mature protein, demonstrating that the signal peptide sequence is genuine. The 248 codons after the signal sequence encode a 185-amino acid extracellular domain consisting of two regions characteristic of the immunoglobulin superfamily, followed by 24-amino acid residues comprising the transmembrane domain and a 39-amino acid cytoplasmic domain. The 248-amino acid residues of the mature protein correspond to an approximate molecular weight of 27,000. However, the purified protein has a larger molecular weight of ~58,000 (13). This difference is mainly due to glycosylation of the protein[7] (20).

We have shown previously that EMMPRIN is present at the surface of tumor cells (13) and has the properties of a membrane-intercalated protein (12). On the basis of these findings, we proposed previously that EMMPRIN is attached to the plasma membrane via a transmembrane domain and interacts with a receptor on fibroblasts via an extracellular domain (1). The presence in the cDNA of sequences typical of a signal peptide and a transmembrane region is consistent with EMMPRIN being an integral plasma membrane protein.

After the termination codon, the cDNA contains a 3′ untranslated region ending in a poly(A) tail. Northern blot analysis indicates that the mRNA for EMMPRIN is ~1.7 kb in size, which is approximately the same as that of the fused EMMPRIN cDNA. It is evident, however, that a portion of the 5′ untranslated region is lacking from this cDNA.

The EMMPRIN cDNA sequences were used in computer searches of the EMBL and GenBank data bases to detect homology with other known proteins. These searches revealed that the EMMPRIN cDNA is identical to two other human cDNAs, encoding proteins of un-

[9] H. Guo, M. Gordon, B. P. Toole, and C. Biswas, Recombinant human tumor cell EMMPRIN stimulates fibroblast metalloproteinase production, manuscript in preparation.

known function, basigin (19) and the M6 antigen (20). Therefore, our studies, which have been performed from a functional standpoint over the course of many years (4, 6, 12–16), have elucidated at least one biological function of these molecules. We are changing our former designation of the molecule from TCSF to EMMPRIN and would suggest that all the above proteins now be designated EMMPRIN, because the acronym more accurately implies at least one definitive function of the glycoprotein, *i.e.*, stimulation of MMP synthesis via cell-cell interaction.

The fact that EMMPRIN is a member of the immunoglobulin superfamily is also compatible with the idea that, in similar fashion to the N-CAM, I-CAM, and other related subgroups of the immunoglobulin superfamily (28), it acts via cell-cell interactions (1). We are currently attempting to identify the molecule on the surface of fibroblasts that interacts with tumor cell-derived EMMPRIN, causing increased fibroblast MMP production. Our recent finding that EMMPRIN is expressed in keratinocytes and localized in the basal layers of the epidermis[7] suggests the possibility that EMMPRIN may have a natural function in embryonic development or wound healing by causing dermal fibroblasts to increase their MMP production, thus facilitating tissue remodeling (18). The antibody to M6 antigen localizes EMMPRIN to granulocytes in patients with rheumatoid arthritis (20), possibly indicating a role for EMMPRIN in stromal MMP production and the consequent matrix degradation that occurs in the arthritic joint. Thus, we propose that EMMPRIN and related molecules are important mediators of matrix remodeling in normal and pathological tissues.

With respect to tumorigenesis, it has become clear that: (*a*) MMPs are crucial to the process of tumor cell invasion through basement membranes and interstitial matrices (1–3); and (*b*) in the case of interstitial collagenase, stromelysin, and M_r 72,000 gelatinase (type IV collagenase), the MMPs involved are produced mainly by peritumoral fibroblasts rather than by the tumor cells themselves (8–11). Since tumor cell-derived EMMPRIN causes a significant increase in the levels of these three enzymes in human fibroblasts (6, 13–16) and since EMMPRIN is associated with the surface of many types of tumor cells *in vivo* and *in vitro* (13, 17),[10] it is very likely that EMMPRIN is a central factor in the stimulation of MMPs required for tumor invasion and metastasis.

ACKNOWLEDGMENTS

We thank Drs. Marion Gordon and Bryan Toole for their helpful suggestions and encouragement during various parts of the project and preparation of the manuscript; Dr. Nicholas Grammatikakis for suggesting the name EMMPRIN; Dr. William S. Lane for performing the amino acid sequencing; Frank Igoe for preparation of primer E; and Diane H. Silva for her technical help and assistance during preparation of the manuscript.

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Communication

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 1, Issue of January 3, pp. 24–27, 1997

Stimulation of Matrix Metalloproteinase Production by Recombinant Extracellular Matrix Metalloproteinase Inducer from Transfected Chinese Hamster Ovary Cells*

(Received for publication, August 13, 1996, and in revised form, October 16, 1996)

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Many of the tumor-associated matrix metalloproteinases that are implicated in metastasis are produced by stromal fibroblasts within or surrounding the tumor in response to stimulation by factors produced by tumor cells. In this study we transfected Chinese hamster ovary cells with putative cDNA for human extracellular matrix metalloproteinase inducer (EMMPRIN), a transmembrane glycoprotein that is attached to the surface of many types of malignant human tumor cells and that has previously been implicated in stimulation of matrix metalloproteinase production in fibroblasts. We show that these transfected cells synthesize EMMPRIN that is extensively post-translationally processed; this recombinant EMMPRIN stimulates human fibroblast production of interstitial collagenase, stromelysin-1, and gelatinase A (72-kDa type IV collagenase). We propose that EMMPRIN regulates matrix metalloproteinase production during tumor invasion and other processes involving tissue remodeling.

Successful tumor metastasis requires many steps, one of which is local proteolytic destruction of extracellular matrix at sites of tumor invasion. A major class of proteinases associated with tumor invasion is the matrix metalloproteinases (MMPs)[1] (1, 2). Although it was initially thought that these enzymes were mainly produced by malignant tumor cells themselves, it is now clear that interstitial collagenase (MMP-1), gelatinase A (MMP-2, a 72-kDa type IV collagenase), and stromelysin-1 (MMP-3) are produced *in vivo* by stromal fibroblasts associated with several types of tumors (2–7). MMP-2 synthesized and secreted by these fibroblasts has been shown to adhere to the surface of tumor cells, facilitating tissue invasion (8–10). Because quiescent fibroblasts generally produce relatively low amounts of MMPs (11, 12), tumor-associated fibroblasts must be influenced in some way to give rise to the elevated levels of MMPs usually present in malignant tumors. One possibility that we have investigated is that tumor cells interact with fibroblasts via soluble or cell-bound factors, stimulating fibroblast MMP production (11–15). Our studies have led to characterization of a tumor cell surface protein, extracellular matrix metalloproteinase inducer (EMMPRIN; previously termed tumor cell-derived collagenase stimulatory factor or TCSF), that stimulates fibroblast production of MMP-1, MMP-2, and MMP-3 (12–14). We recently obtained cDNAs for human EMMPRIN and verified their identity by recognition of recombinant EMMPRIN by activity-blocking monoclonal antibody and by sequence identity with amino acid sequences of peptides isolated from EMMPRIN (15). However, recombinant EMMPRIN produced by bacteria is much smaller than native EMMPRIN isolated from tumor cells because it is not post-translationally processed. This form of recombinant EMMPRIN is inactive, thus leaving some doubt regarding the identity of the cDNAs. In this study, we use the cDNAs to transfect CHO cells and show that EMMPRIN produced by these transfected cells is post-translationally processed and stimulates fibroblasts to produce elevated levels of MMP-1, MMP-2, and MMP-3.

EXPERIMENTAL PROCEDURES

Stable Transfection of CHO Cells with EMMPRIN cDNA—EMMPRIN cDNA (15) was subcloned into an expression vector, pcDNA/Neo (Invitrogen, San Diego, CA), and purified by CsCl gradient centrifugation and phenol/chloroform extraction. CHO cells (American Type Culture Collection, Bethesda, MD) were seeded at 10^6 cells/100-mm tissue culture dish and incubated overnight, at which stage they were 50–70% confluent. The cells then were transfected in 5 ml of serum-free Ham's F-12 medium containing lipofectamine-DNA complex (10 μl of lipofectamine (Life Technologies, Inc.) mixed with 10 μg of DNA with or without the EMMPRIN insert). After 6 h of incubation at 37 °C, 5 ml of medium containing 20% fetal bovine serum was added to the transfection mixture, which was then cultured at 37 °C for a further 72 h. The cells then were treated with trypsin-EDTA (Life Technologies, Inc.) and subcultured in medium containing 400 mg/liter of Geneticin (Life Technologies, Inc.) for 2–3 weeks. Successful transfection was assessed by immunocytochemistry using monoclonal antibody E11F4 raised against EMMPRIN, as described previously (13).

Purification of EMMPRIN—EMMPRIN was purified from detergent extracts of cell membranes from LX-1 cells or stably transfected CHO cells by immunoaffinity chromatography using monoclonal antibody E11F4 against EMMPRIN as described previously (13). Briefly, the cell membranes were extracted with 10 mM Tris-HCl buffer (pH 8.2), containing 0.5% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA. The supernatant of the extract was then applied to a 5-ml anti-EMMPRIN affinity column and recirculated through the column for 12 h at 4 °C. The column was washed with buffer several times, and EMMPRIN was then eluted from the column with 50 mM diethylamine, 30 mM octylglucoside (pH 11.5). The eluted protein was neutralized with 0.5 M NaH_2PO_4, dialyzed against 0.1 M acetic acid, concentrated, and dissolved in 0.1 M acetic acid.

Assays for EMMPRIN Activity—Human fibroblasts (isolated from

* This work was supported by National Institutes of Health Grants CA 38817 (to C. B.) and EY 09056 (to M. K. G.), U. S. Army Medical Research Grant DAMD 17-95-1-5017 (to S. Z. and B. P. T.), and a Merit Review Grant from the Department of Veterans Affairs (to S. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† This article is dedicated to the memory of our friend and colleague, Chitra, in whose laboratory much of this work was done but who died in August, 1993.

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[1] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate ester; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis.

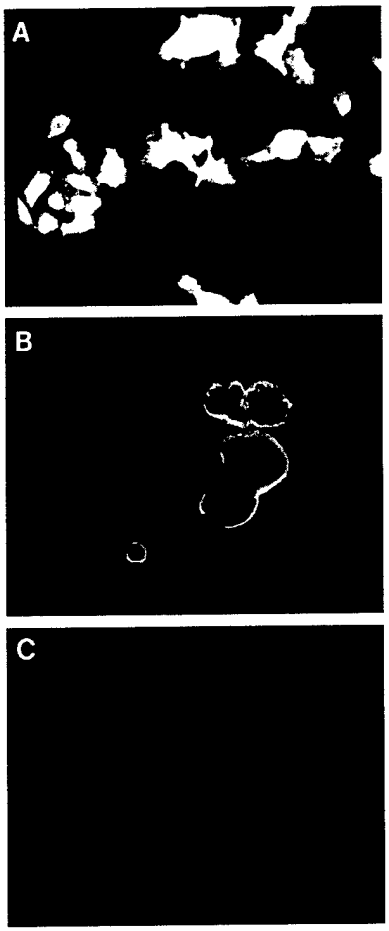

FIG. 1. **Immunofluorescent staining of CHO cells transfected with EMMPRIN cDNA.** Staining was carried out using monoclonal antibody, E11F4, against EMMPRIN as described previously (13). *A*, CHO cells transfected with EMMPRIN cDNA, fixed under normal culture conditions. *B*, similar cells to those in *A*, but fixed 4 h after plating. *C*, CHO cells mock-transfected with vector. Untransfected CHO cells also show no reactivity with E11F4 (not shown).

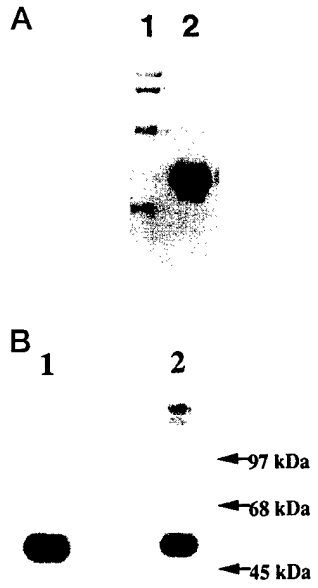

FIG. 2. **SDS-PAGE and Western blotting of purified recombinant EMMPRIN.** *A*, silver-stained SDS-PAGE gel of EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA. EMMPRIN was purified from cell membranes as described under "Experimental Procedures," dissolved in SDS sample buffer containing 0.1 M dithiothreitol, heated at 95 °C for 10 min, and subjected to 10% SDS-PAGE; the gel was deliberately overloaded to reveal potential contaminants. *Lane 1*, molecular mass standards (45, 66, 97, and 116 kDa); *lane 2*, purified recombinant EMMPRIN. *B*, Western blot of recombinant EMMPRIN purified from CHO cells transfected with EMMPRIN cDNA (*lane 1*) and of native EMMPRIN purified from LX-1 cells (*lane 2*). A 10% SDS-PAGE gel was electroblotted to a nitrocellulose membrane followed by blocking with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20. The blot was incubated with E11F4 hybridoma supernatant (13) for 1 h at room temperature and then with horseradish peroxidase-conjugated anti-mouse IgG. The EMMPRIN protein bands were detected with ECL Western blotting detection reagents (Amersham Corp.). In both cases the anti-EMMPRIN antibody recognized a protein with a molecular mass of ~58 kDa. Some immunoreactive, aggregated protein was also present in LX-1 cells, as previously noted (14).

human skin in our laboratory) were cultured for 24 h in 24-well plates in 1 ml of DMEM medium supplemented with 10% fetal bovine serum, after which the medium was replaced with 0.5 ml of DMEM containing 2% fetal bovine serum in the presence or the absence of EMMPRIN or TPA, and the cultures were further incubated at 37 °C for 3 days. Media from these cultures were used for zymographic assay of MMP-3 (16) and ELISA of MMP-1, MMP-2, and MMP-3 (12, 17).

RESULTS

In initial attempts to demonstrate recombinant EMMPRIN activity we tested purified, pGEX bacterial expression protein. However, EMMPRIN produced in the pGEX system had a molecular mass of only ~29 kDa (equivalent to that expected from the cDNA open reading frame (15)), compared with native EMMPRIN from tumor cells, which is ~58 kDa (12–14). This bacterially produced recombinant EMMPRIN protein was inactive in stimulating MMP production by human fibroblasts. Next, COS and CHO cells were transfected with EMMPRIN cDNA under a variety of conditions, but in most cases the EMMPRIN produced was either of similar molecular mass to bacterial recombinant protein, *i.e.* ~29 kDa, or was partially post-translationally processed with molecular masses ranging from 30–45 kDa. EMMPRIN isolated from these cells was also inactive.

However we found that after stable transfection (see "Experimental Procedures"), CHO cells could be selected that synthesize high levels of EMMPRIN of similar molecular mass to that of native EMMPRIN isolated directly from LX-1 human carcinoma cells. Fig. 1*A* shows detection of this recombinant EMMPRIN in the transfected CHO cells by immunocytochemistry using monoclonal antibody raised against native EMMPRIN from LX-1 cells (13). Because the transfected cells are very flat, it is difficult to discern the precise cellular distribution of EMMPRIN. However, if the cells are fixed shortly after plating (~4 h), *i.e.* before they have flattened, it is clear that EMMPRIN is located at the surface of the transfected cells (Fig. 1*B*). Untransfected cells or cells that are mock-transfected with vector only show no reactivity with the antibody (Fig. 1*C*).

Fig. 2*A* shows a silver-stained SDS-PAGE gel of recombinant EMMPRIN purified by immunoaffinity chromatography from membrane extracts of the stably transfected CHO cells; this gel was deliberately overloaded to reveal potential contaminants in the preparation. A single broad band at ~58 kDa was detected, as previously obtained for tumor cell-derived EMMPRIN (13, 14). Direct comparison of purified recombinant and LX-1 carcinoma cell-derived EMMPRIN by Western blotting showed that they were identical in size (Fig. 2*B*), indicating that the recombinant EMMPRIN was fully or almost fully post-translationally processed. Untransfected CHO cells or cells transfected with vector only did not produce any EMMPRIN detectable by immunoaffinity chromatography and Western blotting (not shown).

We then tested purified recombinant EMMPRIN from transfected CHO cells for its ability to stimulate MMP production by human fibroblasts in culture. We first measured the effect of recombinant EMMPRIN on MMP-3 production by zymography,

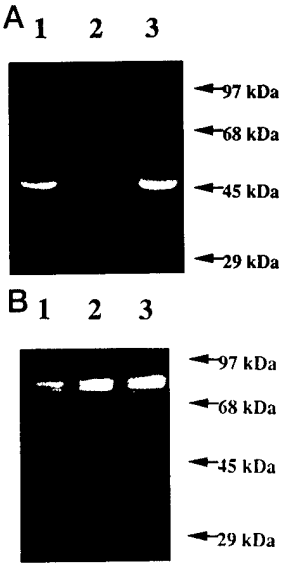

FIG. 3. **Stimulation of stromelysin-1 (MMP-3) production in human fibroblasts incubated with recombinant EMMPRIN isolated from transfected CHO cells.** Media (15 μl each) from fibroblasts cultured in the presence or the absence of EMMPRIN or TPA were subjected to electrophoresis without reduction in 10% SDS-polyacrylamide impregnated with 0.5 mg/ml β-casein (*A*) or 0.5 mg/ml carboxymethylated transferrin (*B*). After electrophoresis, the SDS was eluted from the gels in 2.5% Triton X-100 for 30 min at 25 °C, and the gels were then incubated 20 h in substrate buffer containing 50 mM Tris-HCl and 5 mM $CaCl_2$. The gels were stained with Coomassie Blue, and the presence of proteolytic enzymes was identified by the appearance of clear zones where the casein or transferrin substrate had been digested. *Lane 1*, fibroblasts incubated with recombinant EMMPRIN (100 μg/ml); *lane 2*, fibroblasts incubated without added protein; *lane 3*, fibroblasts incubated with TPA (0.1 μg/ml). The clear band at ~45 kDa present in *lanes 1* and *3*, but in much lower amount in *lane 2*, represents MMP-3. The other clear zones are due to other proteolytic enzymes constitutive to the fibroblasts.

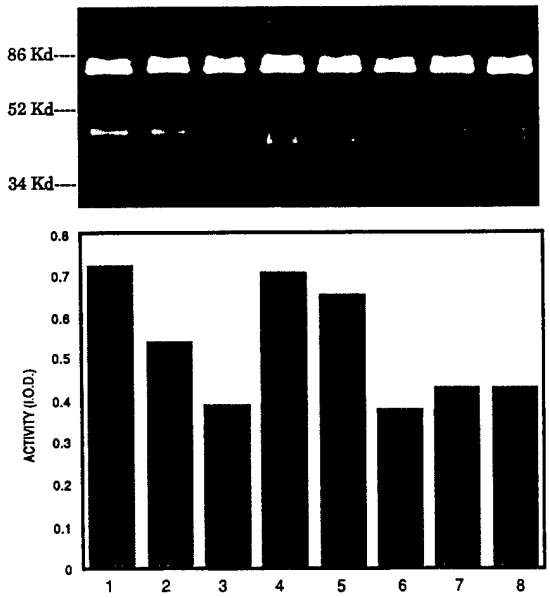

FIG. 4. **Effect of antibody against native EMMPRIN on stimulation of MMP-3 production by recombinant EMMPRIN.** MMP-3 was detected in the same fashion as described in the legend to Fig. 3*B* using 0.5 mg/ml of carboxymethylated transferrin as substrate. The *top panel* shows the zymogram; the *bottom panel* shows quantitation of the ~45-kDa band (or doublet) in each lane by densitometry using a Bio Image Whole Band Analysis package. *Lane 1*, plus 100 ng/ml TPA; *lane 2*, plus 50 ng/ml TPA; *lane 3*, no addition; *lanes 4* and *5*, plus 100 μg/ml recombinant EMMPRIN; *lane 6*, plus supernatant derived after protein A treatment of medium containing 100 μg/ml recombinant EMMPRIN and EIIF4 monoclonal antibody against EMMPRIN, as described previously (13); *lane 7*, plus 100 μg/ml recombinant EMMPRIN and antibody EIIF4; *lane 8*, no addition.

using two separate substrates, casein (Fig. 3*A*) and carboxymethylated transferrin (Fig. 3*B*). A clear-cut increase in active MMP-3 was observed in fibroblasts treated with the recombinant EMMPRIN (Fig. 3, *A* and *B*, *lane 1 versus 2*). The amount of MMP-3 was similar to that induced by TPA treatment (Fig. 3, *A* and *B*, *lane 3*).

To ensure that stimulation of MMP-3 production was not due to minor contaminants in the recombinant EMMPRIN preparation, we tested the effect of blocking antibody raised against native EMMPRIN (13) on the stimulation by recombinant EMMPRIN, using two different approaches. In the first approach, antibody was included in the culture medium together with EMMPRIN throughout the 3-day incubation period (Fig. 4, *lane 7*). In the second approach, the antibody was mixed with EMMPRIN and then removed by binding to protein A; the supernatant from this reaction, depleted of antigen, was then added to the culture for the 3-day incubation (Fig. 4, *lane 6*). As shown in Fig. 4, the stimulation of stromelysin production in cells treated with EMMPRIN (*lanes 4* and *5 versus lanes 3* and *8*) was completely reversed by either of the two different treatments with antibody to EMMPRIN (*lanes 6* and *7*).

Finally, we measured the effect of recombinant EMMPRIN on MMP-1, MMP-2, and MMP-3 production by ELISA. In two separate experiments, treatment of fibroblasts with the EMMPRIN gave rise to significant increments in production of MMP-1 (~6- and ~11-fold), MMP-2 (~1.5- and ~16-fold), and MMP-3 (~2- and ~4-fold) (Table I). In most cases the degree of stimulation of MMP by recombinant EMMPRIN was similar to that caused by TPA (Table I).

DISCUSSION

The results presented here constitute final proof that the cDNAs that we have obtained genuinely encode EMMPRIN, as defined by its MMP stimulatory activity. The degree of stimulation of MMP-1, MMP-2, and MMP-3 by recombinant EMMPRIN obtained herein is similar to that obtained previously with native EMMPRIN purified from LX-1 carcinoma cells (12). It should be pointed out, however, that the degree of stimulation by either native or recombinant EMMPRIN varies in different fibroblast preparations. First, some fibroblast preparations are not significantly responsive to EMMPRIN stimulation, whereas others are very responsive (*e.g.* see Ref. 12). Second, among populations of fibroblasts that are responsive to EMMPRIN, an important variable is the extent to which a batch of fibroblasts already produces a given MMP without addition of stimulatory agents. This can be readily appreciated for MMP-3 production in Fig. 3*B*, *lane 2*, versus Fig. 4, *lanes 3* and *8*, and for MMP-2 production in Table I, experiment 1 *versus* experiment 2. Our overall experience has been that EMMPRIN stimulation of MMP-1 and MMP-3 production is usually in the range of 3–10-fold. Stimulation of MMP-2, however, is even more variable (*e.g.* see Table I, experiment 1 *versus* 2) but is usually rather modest, *e.g.* 1.5–2-fold. One reason for this variability is clearly the significant amounts of MMP-2 that many fibroblast preparations produce without treatment with exogenous agents, *e.g.* experiment 1 in Table I. Also, however, the mechanism of stimulation of MMP-2 may be more complex than for MMP-1 and MMP-3. As well as stimulating overall MMP-2 production, EMMPRIN apparently enhances activation of MMP-2, and this sometimes leads to underestimation of MMP-2 stimulation in ELISA assays (12).

The experiments described here indicate that EMMPRIN activity is dependent on post-translational processing. How-

TABLE I

Stimulation of MMP production by recombinant EMMPRIN

Recombinant EMMPRIN was purified from membranes of transfected CHO cells and, in two separate experiments, added at 100 μg/ml to cultures of human fibroblasts. Cultures were also incubated with TPA (0.1 μg/ml) or with no added reagent. After incubation, aliquots of culture medium were used for ELISA of MMP-1, MMP-2, and MMP-3. Amounts of MMP are expressed as μg/ml ± S.E.

Agent added	MMP-1	MMP-2	MMP-3
Experiment 1			
None	0.03 ± 0.00	1.40 ± 0.01	0.21 ± 0.01
rEMMPRIN	0.33 ± 0.02[a]	2.12 ± 0.13[a]	0.93 ± 0.13[a]
TPA	0.32 ± 0.02[a]	2.33 ± 0.29[a]	0.42 ± 0.02[a]
Experiment 2			
None	0.03 ± 0.00	0.13 ± 0.01	0.35 ± 0.04
rEMMPRIN	0.17 ± 0.02[a]	2.10 ± 0.37[a]	0.63 ± 0.03[a]
TPA	0.25 ± 0.02[a]	0.46 ± 0.06[a]	0.56 ± 0.07[a]

[a] Significantly greater than control (none added), $p < 0.05$.

ever, it is not yet clear whether processing is required to attain the appropriate conformation for activity or whether specific side groups, *e.g.* carbohydrate or phosphate, are involved in EMMPRIN receptor recognition.

We have shown by immunocytochemistry that EMMPRIN is present on the surface of tumor cells but not fibroblasts and several other normal adult cell types (13, 18, 19). Prior studies have also shown that tumor cells shed EMMPRIN (13) and that EMMPRIN appears in the urine of bladder carcinoma patients (18, 20). Thus tumor cell EMMPRIN, in soluble or membrane-bound form, is likely to be responsible for at least part of the stimulation of fibroblast MMP production observed *in vivo* in association with a variety of malignant tumors (3–7). In preliminary experiments, we have found that production of the tissue inhibitor of matrix metalloproteinases, TIMP-1, is not stimulated by EMMPRIN; if this proves to be true *in vivo*, an imbalance of active *versus* inactive MMP production may result from EMMPRIN action on stromal fibroblasts.

The amino acid sequence of EMMPRIN is identical to that of human M6 antigen (21) and basigin (22), for which no function had previously been ascribed until EMMPRIN was cloned (15). Several proteins with high levels of homology to EMMPRIN, *i.e.* neurothelin, HT7, OX47, and gp42, have also been characterized in other species (21–23), but again their function has not been determined. These proteins may be species homologues of one another or closely related members of a protein family; it remains to be seen whether all of these proteins have EMMPRIN activity.

The ability of EMMPRIN to stimulate MMP production has led us to propose that it may be involved in a wide range of physiological and pathological processes where tissue remodeling takes place (24). For example, its presence in the epidermis (25) and in several embryonic epithelia[2] suggests that EMMPRIN may participate in epithelial-mesenchymal interactions leading to changes in tissue architecture during development and wound healing. Also, EMMPRIN on the surface of activated lymphocytes and monocytes (21) may contribute to elevated MMP levels found in arthritis. However, association of EMMPRIN-like material with endothelium during formation of the blood-brain barrier and with highly organized epithelia such as retina and kidney tubules (23) suggests that it may have additional functions involving cell-cell interactions.

Acknowledgments—We thank Diane Silva and Michelle Drews for technical help.

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Volume 45(5): 703–709, 1997
The Journal of Histochemistry & Cytochemistry

ARTICLE

Tumor Collagenase Stimulatory Factor (TCSF) Expression and Localization in Human Lung and Breast Cancers

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SUMMARY Tumor cell-derived collagenase stimulatory factor (TCSF) stimulates in vitro the biosynthesis of various matrix metalloproteinases involved in tumor invasion, such as interstitial collagenase, gelatinase A, and stromelysin 1. The expression of TCSF mRNAs was studied in vivo, using in situ hybridization and Northern blotting analysis, in seven normal tissues and in 22 squamous cell carcinomas of the lung, and in seven benign proliferations and in 22 ductal carcinomas of the mammary gland. By in situ hybridization, TCSF mRNAs were detected in 40 of 44 carcinomas, in pre-invasive and invasive cancer cells of both lung and breast cancers. TCSF mRNAs and gelatinase A mRNAs were both visualized in the same areas in serial sections in breast cancers, and were expressed by different cells, tumor cells, and fibroblasts. The histological results were confirmed by Northern blot analysis, which showed a higher expression of TCSF mRNAs in cancers than in benign and normal tissues. These observations support the hypothesis that TCSF is an important factor in lung and breast tumor progression. **(J Histochem Cytochem 45:703–709, 1997)**

KEY WORDS
TCSF
metalloproteinases
tumor invasion

TUMOR INVASION is a multistep process that involves the degradation of basement membrane and interstitial matrix components by proteolytic enzymes. Many data actually support an important role for the matrix metalloproteinases (MMPs) in this proteolytic event. High levels of MMPs have been described in many cancer cell lines that display high invasive capacity (Gilles et al. 1994; Monsky et al. 1994; Taniguchi et al. 1992,1994; Bernhard et al. 1990; Bonfil et al. 1989). Such an observation has been recently extended to a newly discovered member of the MMPs, membrane type matrix metalloproteinase 1 (MT-MMP-1), which has also been shown to be correlated with in vitro invasiveness (Sato et al. 1994; Okada et al. 1995; Gilles et al. 1996). In vivo, MMPs have also been associated with the metastatic progression of many human cancers (Davies et al. 1993; Clavel et al. 1992; Levy et al. 1991; Monteagudo et al. 1990). However, recent in vivo data obtained by in situ hybridization (ISH) have shown that interstitial collagenase, gelatinase A, stromelysins, and MT-MMP-1 are mostly synthesized by fibroblasts localized near tumor cell clusters (Okada et al. 1995; Pyke et al. 1993; Poulsom et al. 1992,1993; Polette et al. 1991,1993,1996; Basset et al. 1990).

The specific detection of MMPs in peritumoral fibroblasts has led to the hypothesis that tumor cells might induce the synthesis of these enzymes implicated in cancer dissemination. In agreement with such an idea, several investigators have demonstrated cooperation between tumor cells and fibroblasts in vitro in the regulation of several MMPs, such as interstitial collagenase (Noël et al. 1993; Hernandez et al. 1985; Biswas 1984,1985; Bauer et al. 1979) and gelatinase A (Ito et al. 1995; Noël et al. 1994). Furthermore, a tumor cell-derived collagenase stimulatory factor (TCSF) also present in tumor cell-conditioned media, was isolated and purified from the plasma membranes of a human lung carcinoma cell line (Ellis et al. 1989). This factor is a glycoprotein of 58 kD and was recently

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Received for publication June 4, 1996; accepted October 23, 1996 (6A3991).

0022-1554/97/$3.30

identified as a member of the immunoglobulin superfamily (Biswas et al. 1995). In addition to enhancing interstitial collagenase synthesis (Nabeshima et al. 1991), purified TCSF stimulates gelatinase A and stromelysin 1 expression by fibroblasts (Kataoka et al. 1993). Immunohistochemical studies employing a monoclonal antibody directed against TCSF have shown that TCSF is localized to the outer surface of cultured lung cancer cell lines (Ellis et al. 1989). The same distribution was seen in tumors of urinary bladder, in which TCSF was detected at the periphery of cancer cells but not in surrounding stromal cells (Muraoka et al. 1993). Furthermore, TCSF was also localized in tumor cells by immunohistochemistry in invasive and in situ ductal breast cancers (Zucker and Biswas 1994).

On the basis of limited information concerning TCSF localization in cancer tissue, the role of TCSF in tumor progression remains unclear. In the present study, to clarify the cell origin of TCSF and to study its role in cancer invasion, we performed in situ hybridization and Northern blot analysis on human lung and breast carcinomas as well as on normal tissues.

Materials and Methods

Source of Tissue

The tissue was obtained from 22 lungs resected for squamous cell carcinomas of Stages I (10 cases), II (eight cases), and III (four cases) according to the TMN classification, from seven normal lung samples, from 22 ductal breast cancers of Grade 1 (four cases), Grade 2 (14 cases), and Grade 3 (four cases) according to the Scarf and Bloom classification, and from seven benign breast proliferations (two fibrocystic disease and five fibroadenoma).

Tissue Preparation

Part of the samples were frozen in liquid nitrogen for Northern blot analysis and the remainder were fixed in formalin and embedded in paraffin for in situ hybridization.

In Situ Hybridization Localization

Tissue sections (5 μm) were deparaffinized, rehydrated, and treated with 0.2 M HCl for 20 min at room temperature, followed by 15 min in 1 μg/ml proteinase K (Sigma Chemical; St Louis, MO) in Tris–EDTA–NaCl, 37C, to remove basic proteins. The sections were washed in 2 × SSC (sodium saline citrate), acetylated in 0.25% acetic anhydride in 0.1 M triethanolamine for 10 min, and hybridized overnight with ^{35}S-labeled (50C) anti-sense RNA transcripts. TCSF cDNA (1700 BP) and gelatinase A (1500 BP) (a gift from G. Murphy; Cambridge, UK) were subcloned into pBluescript II SK+/− plasmid and pSP64, respectively, and used to prepare ^{35}S-labeled RNA probes. Hybridizations were followed by RNAse treatment (20 μg/ml, 1 h, 37C) to remove unhybridized probe and two stringent washes (50% formamide–2 × SSC, 2 hr at 60C) before autoradiography using D 19 emulsion (Kodak; Rochester, NY). Slides were exposed for 15 days before development. The controls were performed under the same conditions, using ^{35}S-labeled sense RNA probes. All slides were counterstained with HPS (hematoxylin–phloxin–safran), mounted, and examined under a Zeiss Axiophot microscope.

In Situ Hybridization Quantitation by Image Cytometry

Quantitation of the number of hybridization grains/μm^2 was performed with the help of an automated image analyzer, the DISCOVERY system (Becton–Dickinson; Mountain View, CA). After thresholding, the number of grains are counted automatically on at least six fields at high magnification (× 500). At this magnification, one field measures 12,688 μm^2. We performed these measurements on six different samples (three lung and three breast carcinomas) in which we found normal, in situ, and invasive areas on the same tissue section. Statistical analyses of TCSF mRNA expression levels were compared using the non-parametric Mann–Whitney *U*-test. Data were expressed as mean of dots/μm^2 ± SEM. p values equal to or less than 0.05 were considered significant.

Northern Blot Analysis

Extraction of total RNA from tissues was performed by RNAzol treatment (Biogenesis; Bournemouth, UK). Ten μg of each RNA was analyzed by electrophoresis in 1% agarose gels containing 10% formaldehyde and transferred onto nylon membranes (Hybond-N; Amersham, Poole, UK). The membrane was hybridized with the cDNA probe encoding TCSF (1700 BP) labeled with ^{32}P using random priming synthesis (5 × 108 cpm/μg) (Dupont de Nemours; Bruxelles, Belgium). The filters were exposed for 1 day. Membranes were rehybridized to a ubiquitous 36B4 gene probe, which served as a control. Signal intensities were recorded using a CD 60 Desaga (Heidelberg, Germany) laser-scanning densitometer and TCSF levels (in arbitrary units) were standardized with their corresponding 36B4 levels to obtain values independent of RNA quantities deposed onto gels. Statistical analyses of TCSF expression levels were compared using the non-parametric Mann–Whitney *U*-test. Data were expressed as mean ± SEM. Differences or similarities between two populations were considered significant when confidence intervals were <95% (p<0.05).

Results

Lung Lesions

By Northern blotting, TCSF transcripts were detected in 18 of 22 carcinomas. Quantitative analysis showed significantly higher (p<0.05) TCSF mRNA expression in lung carcinomas than in peritumoral lung tissues (Figures 1 and 2A). However, no significant differences between the TCSF mRNA levels were found in accordance with the TNM stage (Figure 2A).

With in situ hybridization, pre-invasive and invasive cancer cells were labeled in 18 of 22 tumors examined (the same positive samples as those found by

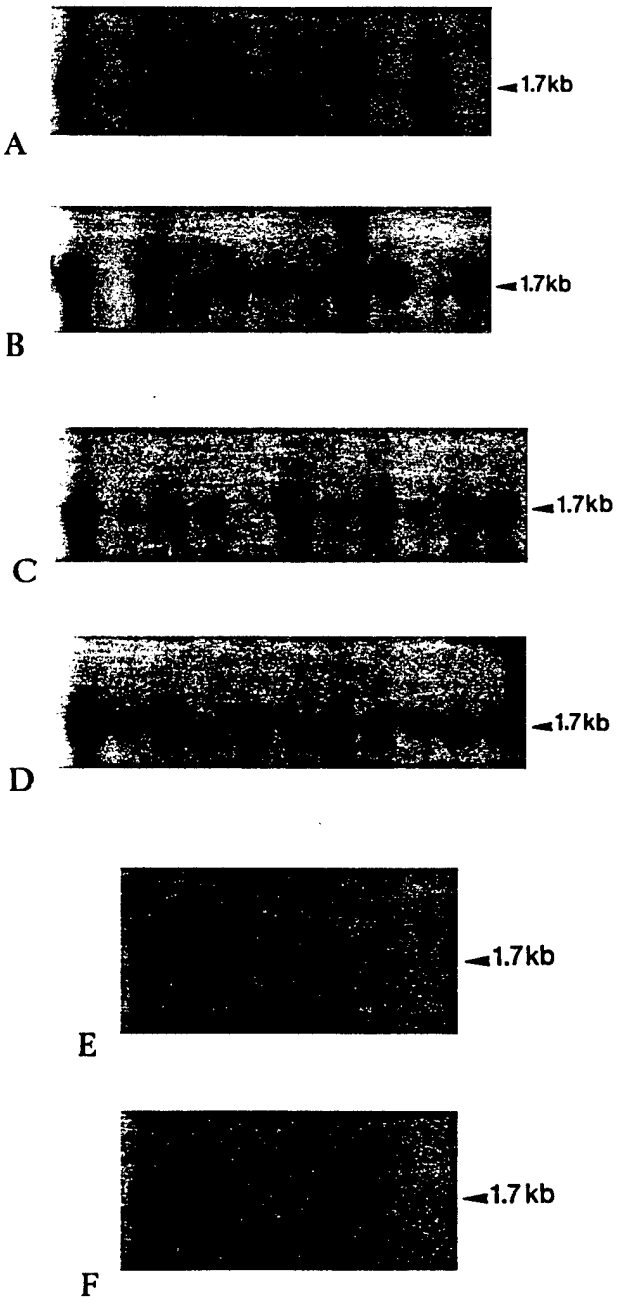

Figure 1 Northern blotting analysis of total RNA extracted from 22 lung carcinomas (**A,B**), 22 breast carcinomas (**C,D**), seven peritumoral normal lung tissue samples (**E**), and seven benign breast proliferations (**F**). A TCSF transcript of 1.7 kb is detected in tumor samples (40/44), whereas it is very weak or undetectable in normal tissue or benign proliferations.

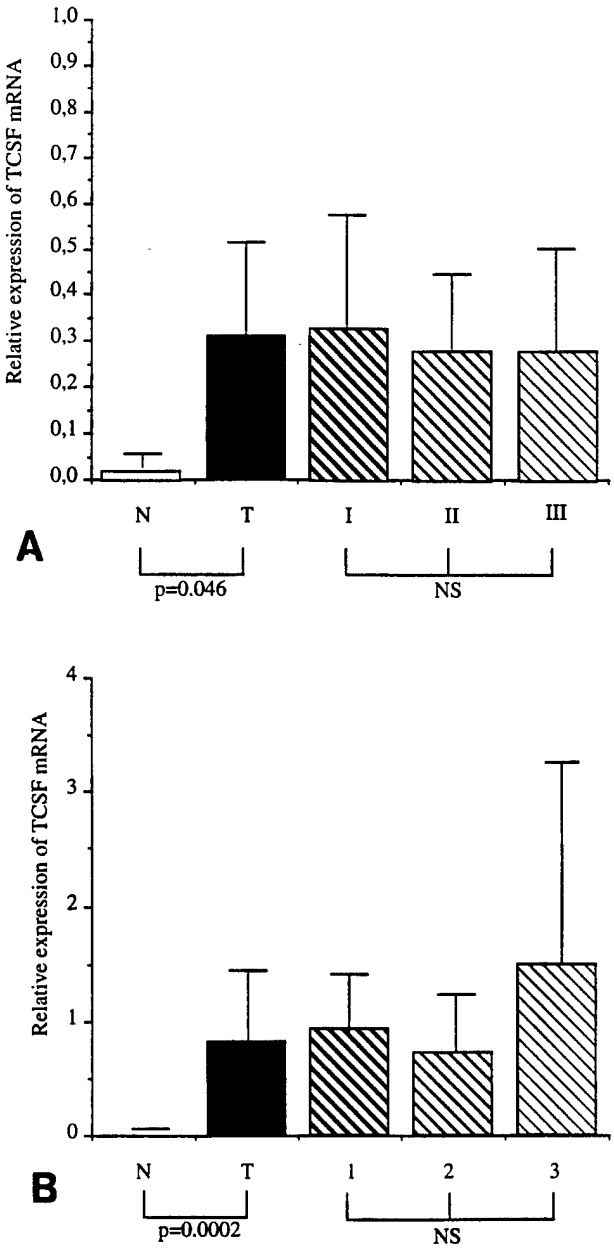

Figure 2 Comparison of TCSF mRNA levels (arbitrary units) according to tissue pathology and to the TNM stage and Scarf and Bloom grading in lung (**A**) and breast (**B**) samples. (**A**) Lung tumor samples (T) expressed significant higher TCSF mRNA levels than non-tumor samples (N). However, no significant differences were found according to the TNM stage of lung carcinomas. (**B**) Breast tumor samples (T) expressed high TCSF mRNA levels whereas no signal was detected in benign breast tissues (N). Statistical analysis did not find any significant differences according to the Scarf and Bloom grade of breast carcinomas.

Northern blot analysis). Stromal cells surrounding labeled invasive cancer cells, were always negative (Figure 3A). Normal (Figure 3C) or squamous metaplastic epithelium and bronchial glands did not express any TCSF transcripts. Moreover, in the normal or emphysematous adjacent lung, pulmonary alveolar macrophages identified by the CD68 monoclonal antibody (Dako; Carpinteria, CA) on serial sections (not shown) were particularly rich in TCSF mRNAs (Figure 3D). In the three cases analyzed by image cytometry, TCSF mRNAs were significantly expressed in tu-

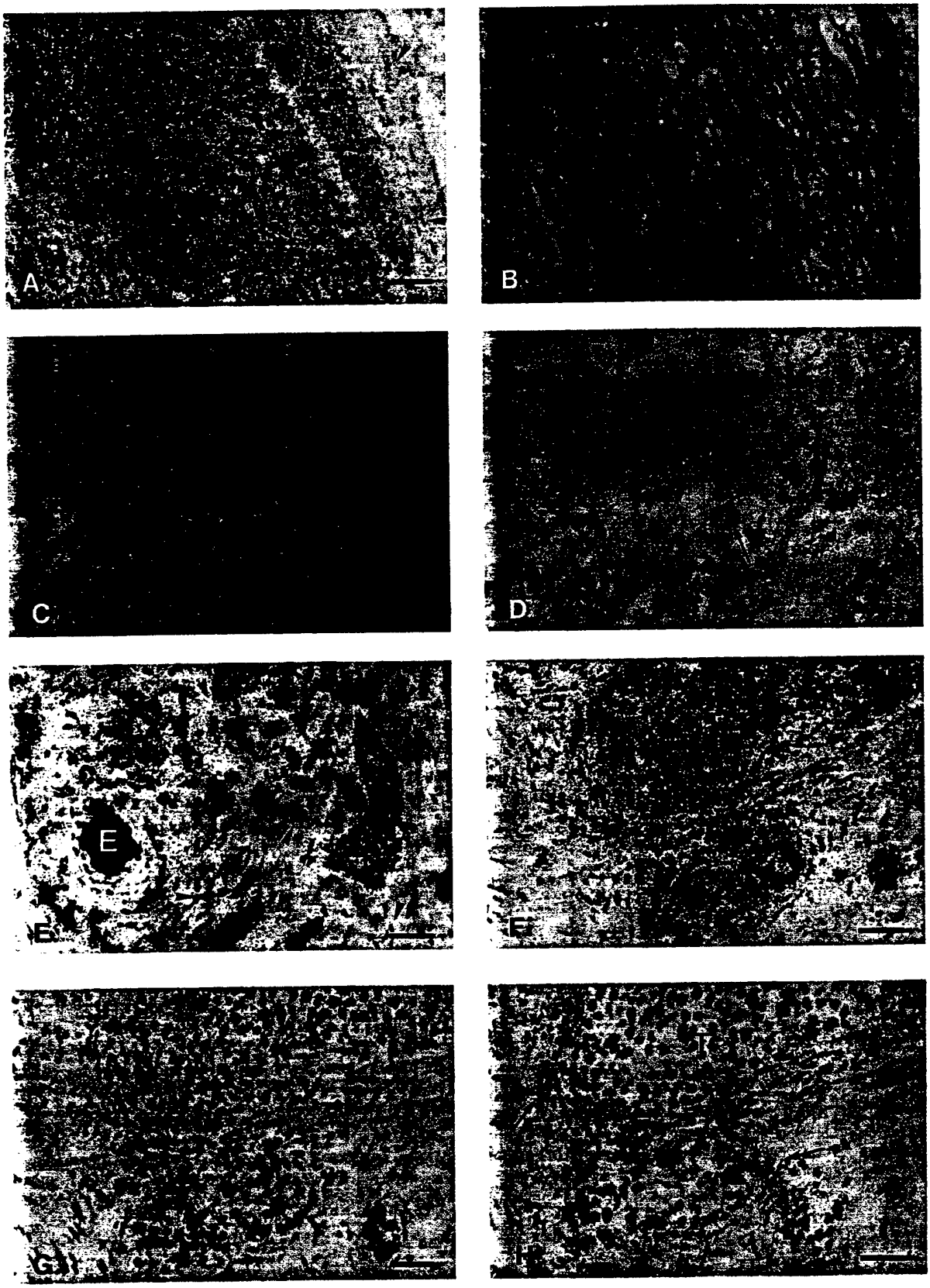

Figure 3 Localization of TCSF in lung cancers and breast cancers. **(A)** TCSF mRNAs are detected in invasive cancer cells (T) in lung carcinoma using an anti-sense probe, whereas stromal cells (arrowheads) are negative. Bar = 70 μm. **(B)** Same area treated with TCSF sense RNA probe. Bar = 70 μm. **(C)** Epithelial cells (E) of normal lung tissue do not express TCSF mRNA. (Bar = 70 μm). **(D)** TCSF mRNA is also present in many alveolar macrophages (arrow). Bar = 70 μm). **(E)** Intraductal breast tumor cells (T) express TCSF mRNAs, whereas epithelial adjacent cells (E) and stromal cells (arrowheads) do not show any hybridization grains. Bar = 70 μm. **(F)** Invasive tumor cells (T) express TCSF mRNAs, whereas stromal cells are negative. Bar = 70 μm. **(G)** Same area treated with TCSF sense RNA probe. Bar = 70 μm. **(H)** Gelatinase A mRNAs are localized in fibroblasts (arrow) in close contact to tumor clusters (T) in serial sections of breast carcinoma. Bar = 70 μm.

mor cell nests of both pre-invasive (1.68 ± 0.22 /μm^2) and invasive areas (2.14 ± 0.35 /μm^2) compared to the extracellular control compartment (0.19 ± 0.02 / μm^2) and normal tissue (0.23 ± 0.04 /μm^2) ($p<0.05$).

Breast Lesions

By Northern blotting, TCSF mRNAs were detected in the 22 breast carcinomas. Quantitative analysis showed significantly higher ($p<0.05$) expression of TCSF mRNAs in breast carcinomas than in benign breast lesions (Figures 1 and 2B). No significant differences between the TCSF mRNA levels were found in accordance with the Scarf and Bloom staging (Figure 2B).

With in situ hybridization, benign proliferations and normal mammary areas mixed with cancer cells or adjacent to cancer areas did not show any hybridization grains (Figure 3E), whereas the TCSF mRNAs were detected in cancer cells in pre-invasive and invasive areas (Figure 3F) of all 22 tumors examined. Stromal cells did not contain any hybridization grains. In the three cases studied by quantification, the density of markers in both pre-invasive (2.14 ± 0.48/μm^2) and invasive (2.95 ± 0.92/μm^2) areas was significantly higher than in the extracellular control compartment (0.23 ± 0.12/μm^2) and the normal areas (0.28 ± 0.10/μm^2) ($p<0.05$). On serial sections, TCSF mRNAs were localized in cancer cells, whereas fibroblasts close to tumor clusters expressed mRNAs encoding gelatinase A (Figures 3F and 3H) in the same areas.

Discussion

In this study we clearly showed the presence of mRNA encoding TCSF in epithelial tumor cells of lung and breast carcinomas. In agreement with our observations, previous immunohistochemical studies detected TCSF in cancer cells in breast (Zucker and Biswas 1994) and in bladder carcinoma (Muraoka et al. 1993). In the latter study, no staining was found in epithelial cells in non-neoplastic urothelium, except in superficial umbrella cells. However, Zucker and Biswas (1994) reported that TCSF is also present in normal breast ductules and lobules near in situ carcinoma areas, describing a more extensive distribution of TCSF compared with our data obtained by in situ hybridization (ISH). No TCSF transcripts were detected in normal epithelial cells in both non-neoplastic breast and lung lesions. It is therefore likely that normal epithelial cells express low levels of the TCSF mRNAs that we could not detect by ISH but that could produce amounts of the TCSF proteins detectable by immunohistochemistry. The presence of TCSF mRNAs in benign and normal tissues was confirmed by our Northern blot analysis, identifying weak expression of TCSF mRNAs in those samples. Furthermore, our Northern blot analysis strengthened our ISH data because a higher significant abundance of TCSF transcripts was found in both breast and lung carcinomas than in benign and normal samples. The detection of TCSF mRNAs in intraepithelial cancer areas of the lung and mammary gland indicates that TCSF mRNA overexpression is an early event in carcinogenesis. These data, taken together, suggest that the expression of TCSF mRNA can be correlated with tumor progression. However, despite its implication in cancer progression, TCSF might play a role in some other pathological processes, such as inflammation and emphysema, because it has also been detected in alveolar macrophages in our study and it has been proposed as a factor in arthritis (Karinrerk et al. 1992).

Even though the precise function of TCSF is not known, some recent in vitro studies have shown that TCSF is able to stimulate the production of several MMPs by fibroblasts. Recent experimental data have demonstrated that TCSF stimulates the production of interstitial collagenase, stromelysin 1 and gelatinase A but not stromelysin 3 in fibroblasts (Kataoka et al. 1993). However, stromelysin 3 is known to have a poor proteolytic activity against collagen-like substrates (Murphy et al. 1994). Moreover, these authors have also demonstrated that TCSF increases activation of gelatinase A. These results are of particular interest regarding the implication of TCSF in cancer progression, because in many carcinomas in vivo the stromal cells have been demonstrated to be the principal source of several MMPs. A variety of studies have indicated that fibroblasts adjacent to the malignant tumors clusters produce interstitial collagenase (Urbanski et al. 1992; Hewitt et al. 1991; Polette et al. 1991), stromelysins 1, 2, 3 (Polette et al. 1991; Basset et al. 1990), and MT-MMP1 (Polette et al. 1996; Okada et al. 1995). More precisely, in both lung and breast carcinomas, mRNAs encoding gelatinase A, which degrades basement membrane collagens (Tryggvason et al. 1993), have also been localized by ISH in the stromal cells surrounding invasive carcinomas (Polette et al. 1993,1994; Poulsom et al. 1992,1993; Soini et al. 1993). In addition to their specific localization at the tumor–stromal interface, MMPs were not or were only weakly found in normal tissues and benign lesions. These studies have therefore demonstrated an association between MMP expression and the invasive process in cancers. Using serial sections in breast carcinomas, we showed that there is an obvious expression of TCSF mRNAs by tumor cells and gelatinase A mRNAs by fibroblasts in the same areas.

It therefore appears that some MMPs, as well as TCSF, are expressed selectively in both pre-invasive and invasive carcinoma but by different cell types, peritumoral fibroblasts and tumor cells, respectively. Relating our in vivo data to the observation that TCSF

enhances the production of some particular MMPs in fibroblasts in vitro, it can be postulated that the TCSF produced by tumor cells in vivo stimulates the expression of some MMPs by peritumoral fibroblasts. However, in vivo, interstitial collagenase and stromelysin 1, which are induced by TCSF in fibroblasts in vitro, are infrequently observed in stromal cells in breast carcinomas (Polette et al. 1991), whereas they are present at high levels in lung cancers (Muller et al. 1991), despite the presence of TCSF in both types of carcinoma. Moreover, in four of 22 of our lung carcinomas, we failed to detect any TCSF transcripts. These findings are consistent with the involvement of other factors in the regulation of those MMPs, such as the origin of the tissue, the extracellular matrix environment, and genetic rearrangements.

In conclusion, our observations on lung and breast cancers strongly support the hypothesis that TCSF is an important factor in tumor progression. More precisely, TCSF produced by tumor cells could play a role in the degradation of extracellular matrix associated with tumor invasion by stimulating the synthesis of some MMPs by peritumoral fibroblasts.

Acknowledgments

We gratefully thank Dr Gillian Murphy for the generous gift of gelatinase A probe.

This material is based on work supported by the US Army Medical Research administration under award no. DAMD 17-95-5017, the ARC no. 1096, and the Lyons Club of Soissons.

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Gene 220 (1998) 99–108

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

Characterization of the gene for human EMMPRIN, a tumor cell surface inducer of matrix metalloproteinases

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Received 24 February 1998; received in revised form 24 June 1998; accepted 1 July 1998; Received by I. Verma

Abstract

EMMPRIN (extracellular matrix metalloproteinase inducer) also known as CD147 and basigin, is a member of the immunoglobulin family that is present on the surface of tumor cells and stimulates nearby fibroblasts to synthesize matrix metalloproteinases. Using our EMMPRIN cDNA, we have isolated a cosmid clone that contains the human EMMPRIN gene. S1 analysis with a fragment of the gene clone and primer extension of the mRNA was performed to determine the transcription start site. PCR and sequence analysis have defined the exon/intron organization of the gene and show that it is highly conserved with the mouse EMMPRIN/basigin gene. About 950 bases of the 5′-flanking region were examined for transcription factor consensus binding sites, locating three SP1 sites and two AP2 sites. The transcription start site was found to be located in a CpG island. Elements in the proximal promoter region were conserved in the human and mouse genes.

Keywords: Tumor cell-derived collagenase stimulatory factor; basigin; M6; CD147

1. Introduction

EMMPRIN (also called CD147, basigin or M6 in the human) is a member of the immunoglobulin superfamily, which includes T cell receptors, neural cell adhesion molecules and major histocompatibility complex antigens. Several groups (Altruda et al., 1989; Ellis et al., 1989; Fadool and Linser, 1993; Kasinrerk et al., 1992; Miyauchi et al., 1990; Nehme et al., 1993; Seulberger et al., 1992) independently discovered this glycoprotein in a variety of species, following different experimental pathways. In the first functional approach, the Biswas laboratory discovered EMMPRIN, then called tumor cell-derived collagenase stimulatory factor (TCSF), as a surface molecule on tumor cells that stimulated nearby fibroblasts to produce matrix metalloproteinases (Biswas et al., 1995; Ellis et al., 1989; Guo et al., 1997; Kataoka et al., 1993). This intercellular recognition function is consistent with the known functions of the immunoglobulin superfamily.

cDNA hybridizations have been used to determine the chromosomal location of both the mouse and human EMMPRIN/basigin genes, mapping the mouse to chromosome 10 (Simon-Chazottes et al., 1992) and the human to 19p13.3 (Kaname et al., 1993). The gene has been disrupted in a transgenic mouse line. Most transgenic embryos lacking the EMMPRIN/basigin gene die around the time of implantation (Igakura et al., 1996; Igakura et al., 1998), a time when intercellular recognition events and matrix metalloproteinase involve-

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[1] Chitra Biswas, our Tufts colleague who started this work, died 26 August 1993.

Abbreviations: AMV, avian myeloblastosis virus; bp, base pair(s); cDNA, DNA complementary to RNA; CpG, deoxycytidyl-deoxyguanosyl dinucleotide; dUTP, deoxyuridyl triphosphate; EDTA, [*N*, *N*, *N*′, *N*′]-ethylenediaminetetraacetic acid; EMMPRIN, extracellular matrix metalloproteinase inducer; FISH, fluorescence in situ hybridization; fmol, femtomol or 10^{-15} mole; Ig, immunoglobulin; kb, kilobase; nt, nucleotide(s); PCR, polymerase chain reaction; rc, reverse complement; SDS, sodium dodecyl sulfate; SSPE, standard saline phosphate-EDTA; TCSF, Tumor cell-derived collagenase stimulatory factor; UTR, untranslated region.

0378-1119/98/$ – see front matter
PII: S0378-1119(98)00400-4

ment are critical (Alexander et al., 1996). In addition, mouse genomic clones have been characterized (Cheng et al., 1994; Miyauchi et al., 1995). In this report we present the first isolation and characterization of the human gene. We have determined the transcription start site and elucidated the exon/intron structure. While a high degree of conservation is seen between the human and mouse gene structures, a notable difference is that the human protein is encoded by eight exons, while that of the mouse is encoded by seven. We have also sequenced approx. 950 bp of the 5′-flanking region of the EMMPRIN gene and have searched it for transcription factor binding site consensus sequences.

2. Materials and methods

2.1. Isolation of the EMMPRIN genomic clone

Filter lifts of a human placental genomic library in cosmid vector pWE15 (Stratagene, La Jolla, CA, USA) were screened with the full-length EMMPRIN cDNA (Biswas et al., 1995), radiolabeled by nick translation. A positively hybridizing candidate cosmid was isolated and expanded. Two criteria were used to verify the cosmid's identity. The first was partial sequence analysis, which indicated that some EMMPRIN sequences were contained within the cosmid clone. The second verification method was fluorescent in situ hybridization (FISH), with biotinylated cosmid clone as probe. Biotin-11-dUTP was incorporated into the probe using the Bio-Nick nick translation kit (Life Technologies, Grand Island, NY, USA), following the manufacturer's instructions. The probe was hybridized in situ to BrdU-synchronized normal female lymphocytes (Lemieux et al., 1992; Lichter et al., 1990), followed by reaction with fluorescein goat anti-biotin and fluorescein-labeled anti-goat IgG (Vector Laboratories, Inc. Burlingame, CA.). Visualization of hybridization to metaphase chromosomes was accomplished by epifluorescence microscopy. The selected cosmid localized only to chromosome 19p13.3 and was used for all subsequent analyses.

To isolate a manageable piece of DNA from the cosmid clone for further analysis, the cosmid DNA containing the EMMPRIN gene was digested with several restriction enzymes. Fragments were separated by electrophoresis on a 1% agarose gel, followed by transfer to nylon membrane. This was hybridized with a ^{32}P-end-labeled 25-mer oligonucleotide probe derived from the EMMPRIN cDNA (CAGCGCGA-ATCCCAGCAGCACGAAC, positions 52–76 in Fig. 1). After hybridization, the blot was washed twice at 65°C with 1×SSPE, 0.1% SDS, dried and exposed to film. A 3 kb *Bam*HI/*Eco*RI double digestion fragment hybridized to the probe. Another aliquot of cosmid DNA was double digested and the 3 kb band was purified from an agarose gel, then subcloned in pBluescript vector (Stratagene). The sequence of this genomic fragment was generated using a Bst DNA sequencing Kit (Bio-Rad, Hercules, CA, USA).

2.2. mRNA isolation, primer extension and S1 nuclease analyses

Total RNA was prepared from LX-1 cells as described previously (Biswas et al., 1995). To determine the transcription start site (the beginning of exon 1), two genomic fragments were prepared that contained the 5′-end of the cDNA (estimated to be approx. 100 nucleotides short of a full-length copy of the mRNA) plus several hundred bases upstream, for hybridizing to the RNA prior to S1 nuclease digestion. The two genomic fragments, 331 nt (−263 to +68 in Fig. 1) and 492 nt (−424 to +68), were amplified from the cosmid template by PCR using the following primers: as 3′-primer for generating both fragments, GATGATGATATC-CCAGCAGCACGAACAG, (an *Eco*RV recognition sequence [in bold], preceded by 6 nt to ensure that the enzymatic site would not be at an end where local strand separation occurs, preventing cleavage, and followed by the reverse complement of nucleotides 49–68 (Fig. 1): bases 66–68 (Fig. 1) overlap the restriction enzyme recognition site); for the smaller product the 5′-primer was nucleotides −263 to −244 (Fig. 1 and Fig. 2A); for the larger product the 5′-primer was −424 to −403 (Fig. 1 and Fig. 2A). These 5′-primers are double underlined in Fig. 1. The PCR products were subcloned into pCRII (Invitrogen, San Diego, CA, USA), and used to transform bacteria. Colonies were grown and selected, and isolated plasmid DNAs were digested with *Eco*RV, and applied to a 1% agarose gel. The linearized products that were extracted from the gel were end-labeled with ^{32}P by T4 polynucleotide kinase. Since the subcloned genomic sequences contained no *Bam*HI sites, but such a site was present in the vector at a site distal from the *Eco*RV site, a subsequent cleavage with *Bam*HI released the entire genomic insert attached to a piece of the vector. In addition, the double digestion ensured that only the strand of genomic DNA complementary to the mRNA contained the label. The large and small double digestion products were gel purified and used for S1 nuclease protection using an S1 Assay kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. Briefly, 50 μg of total RNA was mixed with 50 000 cpm of each probe in hybridization buffer (80% formamide, 100 mM sodium citrate, 300 mM sodium acetate, 1 mM EDTA, pH 6.4), heated at 90°C for 5 min and hybridized at 42°C overnight. The hybridization mixtures were then digested with 50 unit of S1 nuclease for 30 min at 37°C. Following ethanol precipitation, the digested products were resus-

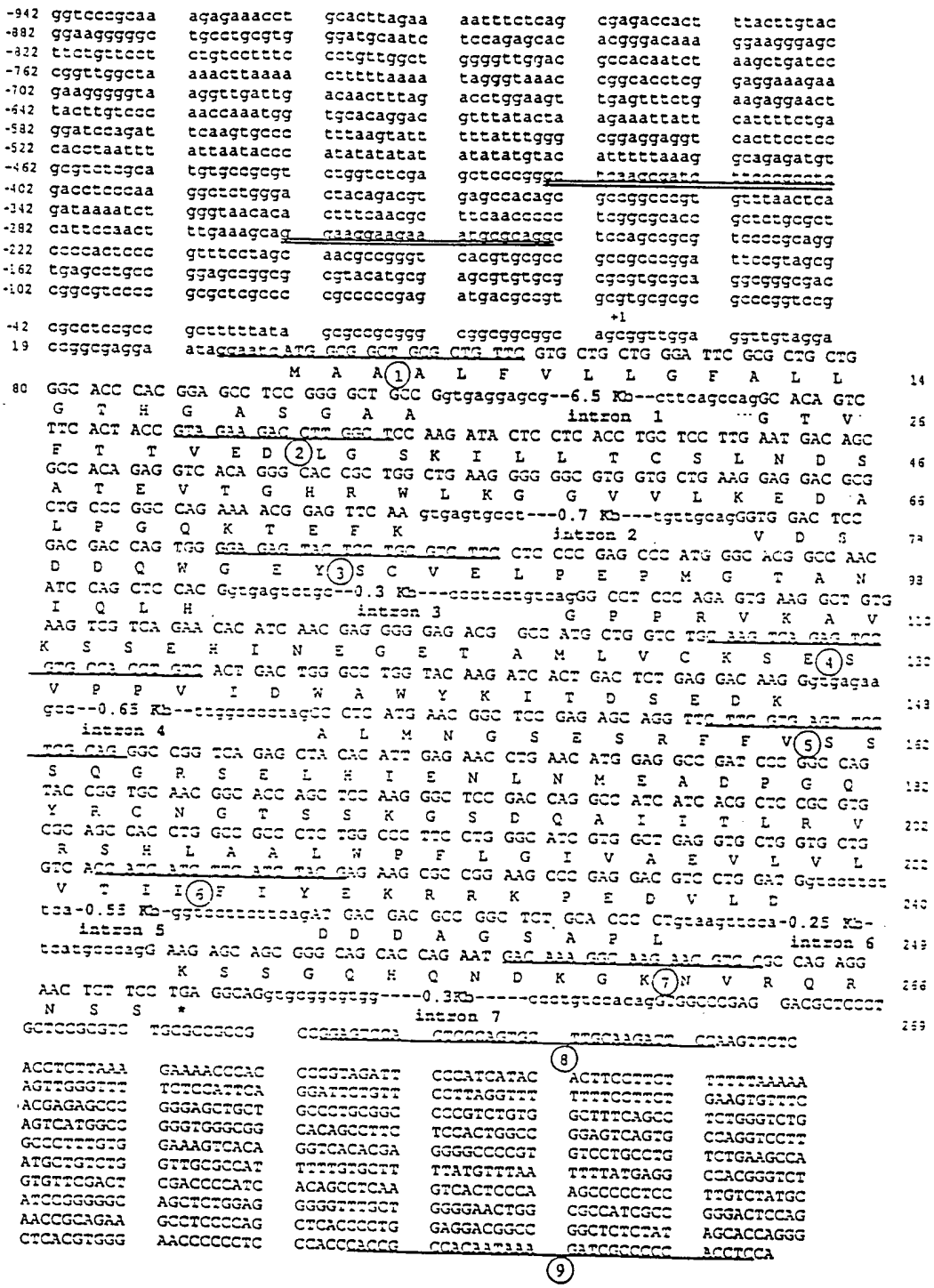

Fig. 1. Sequence of the EMMPRIN gene and 5′-flanking region. A +1 is shown above the transcription start site. The sequence includes 942 bases 5′ of the transcription start site, all the exonic sequences, and the borders of the introns. The 5′-primers used to generate S1 nuclease probes are indicated by double underlines. Primers used to determine the exon, intron organization are singie underlined with circled numbers below them. Exonic sequences are in upper-case letters: intronic and 5′-flanking sequences are in lower-case letters. The stop codon is indicated with an asterisk.

pended in loading buffer (95% formamide, 0.5 mM EDTA, 0.025% SDS, 0.025% xylene cyanol, 0.025% bromophenol blue) and resolved on a 5% polyacrylamide–8 M urea denaturing gel. The gel was dried and exposed to X-ray film.

A negative and positive control probe were also used in the S1 nuclease analysis. Since the Northern blot estimated the mRNA size as approx. 1.6 kb, about 100 bases longer than the cDNA, we reasoned that regions in the gene more than 100 bases upstream of the coding

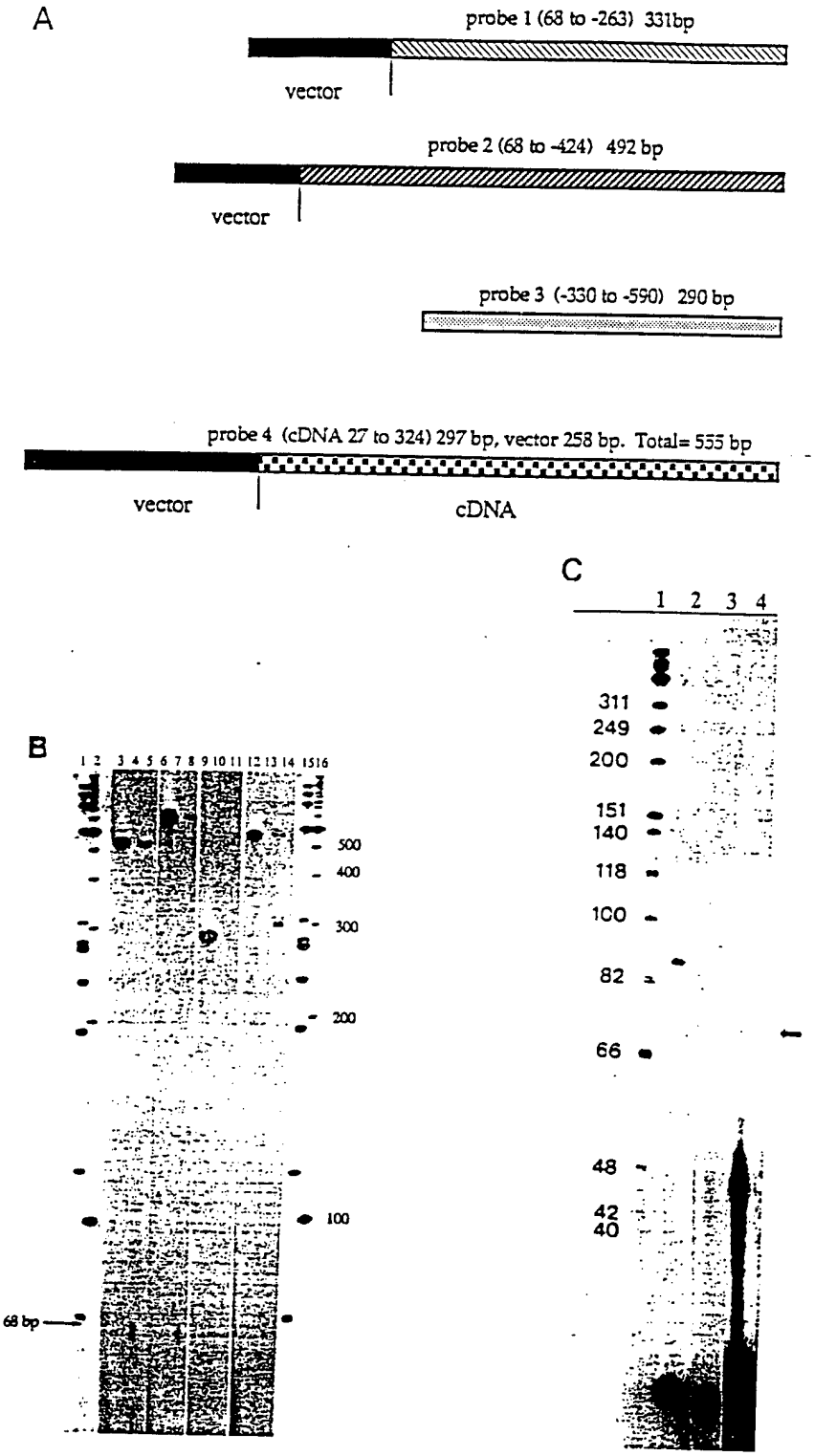

Fig. 2. (A) Probes used for S1 nuclease analysis. Solid black lines indicate vector portions of the probes. Numbering refers to Fig. 1. Probe 3 is a negative control; probe 4 is a positive control. (B) S1 nuclease analysis to determine the transcription start site. Lanes 1 and 15, radiolabeled size marker ϕX174 digested with *Hae*III; lanes 2 and 16, radiolabeled 100 bp ladder size marker; lane 3, the 331 bp EMMPRIN probe with approx. 200 bp vector attached; lane 4, the vector/331 bp probe digested with S1 nuclease; lane 5, the vector/331 bp probe hybridized to mRNA, then digested with S1 nuclease; lane 6, the 492 bp probe with approx. 200 bp vector attached; lane 7, the vector 492 bp probe digested with S1 nuclease; lane 8, the vector/492 bp probe hybridized to mRNA, then digested with S1 nuclease; lane 9, the negative control probe; lane 10, the negative control probe digested with S1 nuclease; lane 11, the negative control probe hybridized to mRNA, then digested with S1 nuclease; lane 12, the positive control probe derived from an internal region of the EMMPRIN cDNA; lane 13, the positive control probe digested with S1 nuclease; lane 14, the positive control probe hybridized to mRNA, then digested with S1 nuclease. Lanes 5 and 8 show that a 68 bp fragment (arrow) of

region would not be in the mRNA and could serve as a negative control. Therefore, a labeled PCR product corresponding to gene nucleotides −330 to −590 (Fig. 1) was labeled as a negative control probe. A positive control, a restriction fragment of a partial cDNA clone, was employed to assure us that the S1 nuclease protection analysis was working perfectly, i.e. that only protected products were observed, one being the 555 bp reannealed probe (since the probes are double-stranded DNA, a portion of the probe is expected to reanneal to itself, thereby preventing digestion), and the other being the probe fragment protected by mRNA. This positive control probe contained the 5′-most 297 bases of our partial copy cDNA (the nucleotide numbering refers to Fig. 1—plus numbers are exonic sequences only), attached to 258 bp of vector (totaling 555 bp, see Fig. 2A).

For primer extension analysis, a Promega Primer Extension System kit was used. 10 pmol of control primer or EMMPRIN antisense primer (GAATCCCAGCAGCACGAACAGCGCA: reverse complement of nucleotides 46–70) were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase for 10 min at 37°C. The reactions were inactivated by heating to 90°C for 2 min, and the primer concentration was adjusted to 100 fmol/μl with water. 100 fmol of each labeled primer was aliquotted for the following ethanol precipitations: with control primer alone (no RNA), with control primer plus 1 μg kit control RNA, with EMMPRIN antisense primer alone (no RNA), and with EMMPRIN antisense primer plus 1 μg LX-1 mRNA. The pellets were resuspended in 10 μl hybridization buffer (80% formamide, 1 mM EDTA, 100 mM sodium citrate, 300 mM sodium acetate, pH 6.4), heated to 95°C for 5 min, then allowed to hybridize overnight at 42°C. These mixtures were again ethanol precipitated then resuspended with 10 μl AMV primer extension buffer (Promega, Madison, WI, USA) containing 1 unit of AMV reverse transcriptase. After incubation for 90 min at 42°C, 10 μl loading buffer (98% formamide, 10 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue) were added and the mixture was heated to 90°C for 10 min. 10 μl of each sample was electrophoretically separated on a 10% denaturing polyacrylamide gel. The gel was dried and exposed to film.

2.3. Determination of exon/intron junctions

Several steps were employed to elucidate the EMMPRIN gene structure. First, to determine the overall size of the gene, a primer derived from the 5′-most end of the cDNA and one from the 3′-most end, just upstream of the polyA addition signal (primers 1 and 9rc, respectively—sequences are given below, and underlined in Fig. 1) were used in PCR to amplify the cosmid template with the Extend long template kit (Boehringer Mannheim, Mannheim, Germany). A 10–11 kb product was amplified. Second, the entire sequence of the subcloned 3.7 kb *Bam*HI/*Eco*RI fragment was determined. The S1 nuclease analysis had defined the 5′-border of exon 1, so this sequencing determined that the 3.7 kb fragment contained approx. 0.95 kb of 5′-flanking sequence. By comparison with the cDNA sequence it also defined the 3′-border of exon 1. The remaining 2.65 kb of 3′-sequence consisted of the beginning of intron 1. Primers 1 and 2rc (underlined in Fig. 1), from exons 1 and 2, were used in PCR with the cosmid clone as template to determine the length of intron 1; sequencing with 2rc defined the sequence at the 3′-end of intron 1, bordering exon 2. The presence of the remaining introns was generally determined by using primer pairs that amplified larger products with cosmid template compared with cDNA template, as assayed on 1 or 2% agarose gels (FMC Corp., Rockland, ME, USA). Intron sizes were estimated by subtracting the genomic product size from the cDNA product size. To define the size of intron 2, a PCR with primers 2 and 3rc was performed; sequencing with these primers determined the intron 2 borders. The same strategy was used with primers 3+4rc for intron 3, 4+5rc for intron 4, and 7+8rc for intron 7. The borders of introns 5 and 6 were more difficult to find. A PCR of the cosmid with primers 5 and 7rc indicated the presence of at least one intron. Sequencing with 7rc defined the 3′-border of intron 6. However, sequencing with primer 5 produced only exonic sequence. Therefore, the 5+7rc PCR product was subcloned into pCRII (Invitrogen). A traditional step-wise sequencing strategy was employed, using the sequence generated by primer 5 to determine what primer (primer 6) to use in the next sequence reaction, and so on. In this way sequencing proceeded through exon 5, intron 5, exon 6, and into the beginning (5′-border) of intron 6 (the primers used to sequence internal regions of intron 5 are not listed as they are not present in Fig. 1). Summation of the determined intron sizes combined with those of the exons yielded the total gene size, 10.8 kb.

Primer sequences (underlined and labeled by a circled number in Fig. 1): Primer 1 = nucleotides 32–55 (as

probes 1 and 2 is protected. Lane 11 shows that the negative control probe is not protected at all by the mRNA. Lane 14 shows that the 297 bp fragment from the interior of the cDNA is protected as expected. (C) Primer extension analysis. Lane 1, radioactively labeled ϕX174+*Hae*III marker; lane 2, kit control primer extension showing the expected 87 nucleotide product reverse transcribed from control mRNA; lane 3, a primer extension reaction using the 25 nucleotide EMMPRIN antisense primer, but no mRNA in the reaction mix; lane 4, a primer extension reaction containing 1 μg of LX-1 mRNA with the 25 nucleotide EMMPRIN antisense primer. A product of 70 nucleotides was produced (arrow). Since the 3′-end of the primer is 33 nucleotides from the A residue of the ATG start codon, the remaining product represents the 5′-UTR (=37 bases).

numbered in Fig. 1) (GGAATCATGGCGGCTGCGCTGTTC); primer 2 = nucleotides encoding amino acid residues 30–35 (GTAGAAGACCTTGGCT); primer 3 = the nucleotides encoding amino acid residues 83–89 (GGAGAGTACTCCTGCGTCTTC); primer 4 = nucleotides (nts) encoding amino acid residues 126–134 (CAAGTCAGAGTCCGTGCCACCTGTC); primer 5 = nts encoding amino acid residues 158–164 (CTTCGTGAGTTCCTCGCAG); primer 6 = nts encoding amino acid residues 258–264 (GACAAAGGCAAGAACGTCC); primer 8 is toward the start of the 3′-untranslated region (see Fig. 1) (GGAGTCCACTCCCAGTGCTTGCAAGATTCC); where the 'rc' designation follows primers 2–8, the sequence is the reverse complement of the sequence given above for that primer; primer 9rc is the reverse complement of nucleotides at the end of the 3′-UTR. Its 5′- to 3′-rc sequence is GAGGTGGGGGCGATCTTTATTGTGGCGGTG.

2.4. 5′-flanking region of the gene

The 3.7 kb *Eco*RI/*Bam*HI genomic fragment isolated from the cosmid clone contained approx. 0.95 kb of sequence upstream of the transcription start site, as determined by sequence analysis. This region was both visually inspected for cis elements and searched for transcription factor binding sites using the Transfac program. Only sequences that conform to the consensus are indicated in the reported sequence.

2.5. Accession numbers

All sequences in Fig. 1 have been submitted to Genbank. They have been assigned accession numbers AF042848, AF042849, AF042850, AF042851, AF042852, AF042853, AF042854, and AF042855.

3. Results

3.1. The EMMPRIN gene clone

A cosmid genomic library was screened with full-length EMMPRIN cDNA to isolate candidate genomic clones. To avoid any possibility of analyzing an artifact cosmid clone, i.e. one generated by ligation of more than one non-contiguous piece of genomic DNA, two criteria were used to assess selected candidates. First, limited sequence analysis revealed that EMMPRIN sequences were contained within the selected positive clone. Second, fluorescent in situ hybridization was used to map the cosmid clone to the correct location on chromosome 19. The metaphase chromosomes of 49 lymphocyte cells were analyzed with the candidate cosmid DNA. Forty-five hybridization signals localized to chromosome 19p13.3, the known location for the EMMPRIN gene (Kaname et al., 1993). No other reproducible hybridization signals were seen. Therefore, the cosmid's genomic DNA represents a single, continuous approx. 30 kb piece of DNA containing the EMMPRIN gene. A 3.7 kb *Eco*RI/*Bam*HI fragment contained the 5′-portion of the gene. PCR amplifications generated the remainder. The genomic primary structure is shown in Fig. 1.

3.2. Determination of the transcription start site

Northern analysis suggested that the full-length mRNA was approx. 1.6 kb. Our cDNA (Biswas et al., 1995) was slightly smaller, missing about 100 nucleotides from the 5′-end. We therefore hybridized fragments of the genomic clone to LX-1 tumor cell mRNA, the source of our EMMPRIN cDNA, and performed an S1 nuclease analysis, to determine the possible transcription start site of the gene, defining the beginning of exon 1. To accomplish this, two genomic fragments of 492 and 331 bp, with identical 3′-ends (at +68 in Fig. 1) were labeled, hybridized to mRNA, and treated with S1 nuclease. As can be seen in Fig. 2B, lanes 5 and 8, 68 bp of both probes were protected. Since the 3′-end of both probes encompasses 31 bases of translated sequence, the total length of the 5′-UTR is 68 minus 31 bases, or 37 bases. No other transcription start sites were suggested by the S1 nuclease analysis.

If the 5′-UTR of the EMMPRIN mRNA was encoded by more than one exon, using a gene fragment as a probe could define an exon/intron junction rather than the true start of exon 1. To rule out this possibility, primer extension analysis was performed. An EMMPRIN-specific antisense primer (reverse complement of nucleotides 46–70 in Fig. 1) was used to reverse transcribe LX-1 cell mRNA. The resulting EMMPRIN cDNA was 70 nucleotides in length (see Fig. 2C). As 33 nucleotides of this product correspond to coding sequence, the remaining 37 correspond to the 5′-UTR. Therefore, both S1 nuclease and primer extension analyses define the start site of transcription (the beginning of exon 1) as that shown by the +1 in Fig. 1.

3.3. The exon/intron structure of the gene

The EMMPRIN gene is encoded by eight exons encompassing 10.8 kb (Figs. 1 and 3). The gene organization is shown in Fig. 3. (The strategy used to arrive at this structure is described in Materials and Methods.) Exon 1 (108 bp) is separated from exon 2 (154 bp) by the approx. 6.5 kb of intron 1, the largest intronic sequence in the EMMPRIN gene. Intron 2 is approx. 700 bp, the second largest intervening sequence in the gene. Exon 3 (83 bp) is separated from exon 4 (138 bp) by the approx. 300 bp intron 3. Intron 4 is approx.

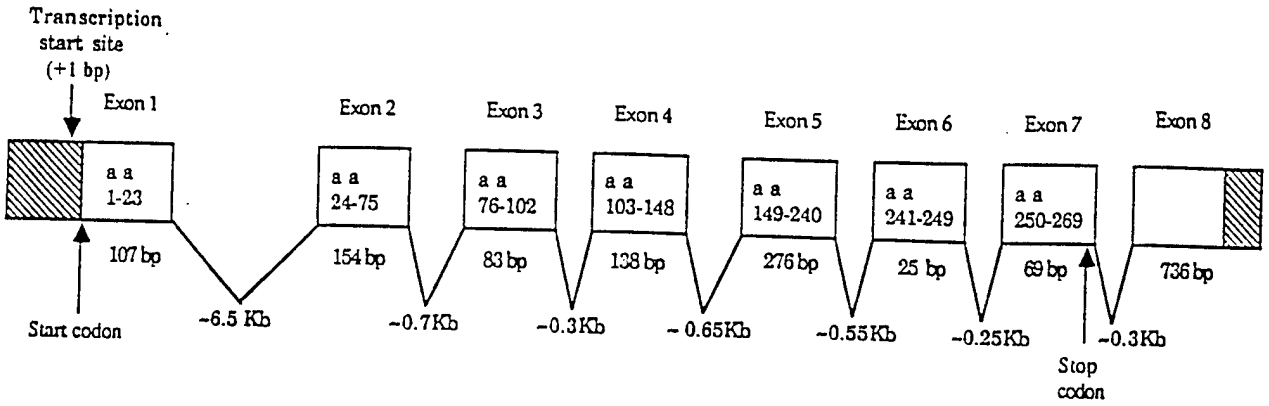

Fig. 3. The EMMPRIN gene organization, outlining the exons and introns. The sum of the exon and intron sizes is 10.8 kb.

650 bp; exon 5 is 276 bp; intron 5 is approx. 550 bp; exon 6 is very short, only 25 bp; intron 6 is approx. 250 bp; exon 7 is 69 bp; intron 7 is approx. 300 bp; and the final exon, exon 8 is 736 bp.

Fig. 4 shows a model of the mRNA, delineating important domains of the protein, and indicating which exons encode these domains. Exon 1 encodes the 5'-untranslated region, the signal peptide and 1 1/3 codons of the first Ig domain. Together, exons 2 and 3 encode the bulk of the first Ig domain; exon 2 encodes 52 codons, or approx. 66% of Ig I; exon 3 encodes 27 codons, or approximately 34% of Ig I. Exons 4 and 5 encode the second Ig domain; exon 4 is 46 codons, comprising approx. 45% of the domain; exon 5 is a 'junctional' exon, encoding the remaining 55% of the Ig domain, as well as the 24 amino acid residues of the transmembrane domain, and a small portion of the intracellular domain. Exons 6 and 7 encode the intracellular domain. Exon 7 also encodes the stop codon and five nucleotide residues of the 3'-untranslated region. Exon 8 encodes the remainder of the 3'-untranslated region.

3.4. The 5'-flanking region of the EMMPRIN gene

Visual inspection of the EMMPRIN gene sequence found no TATA or CAAT boxes in appropriate locations relative to the transcription start site. It did, however, reveal that the transcription start site falls within a CpG island. The occurrence of this dinucleotide is particularly plentiful between nucleotide positions −247 and +6. Transfac computer analysis of the approx. 0.95 kb 5'-flanking sequence revealed the presence of three consensus binding sites for SP1 and two for AP2 (Fig. 5).

4. Discussion

4.1. Comparison of the human exon/intron structure with that of mouse

The gene for mouse EMMPRIN, called basigin, has been localized to chromosome 10 (Simon-Chazottes et al., 1992), and the organization of the mouse gene has been described (Cheng et al., 1994; Miyauchi et al., 1995). The human gene has been mapped by fluorescence in situ hybridization and G-banding on human metaphase chromosomes to 19p13.3 (Kaname et al., 1993), but was not further characterized. Therefore, we decided to isolate and characterize the human EMMPRIN gene structure.

After isolating a cosmid clone containing the gene, we first determined the transcription start site, defining the beginning of exon 1. S1 nuclease analysis revealed that the 5'-UTR of the human EMMPRIN mRNA is 37 bases long. The mouse 5'-UTR was not experimentally determined, but was deduced to be 20 nucleotides from the ATG start codon.

The exon/intron structure of the human gene was determined, and the sizes of the exons and introns were calculated. The overall size of the human gene is larger than the mouse gene. The exons are dispersed in 10.8 kb in the human, and 7.5 kb in the mouse. The sizes of the exons are conserved across the species, therefore the difference in the sizes of the genes is due to smaller introns in the mouse gene. For the most part, the introns for both genes are of ordinary size (700 bp or less) with the exception of the first intron, which is about 4.7 kb in mouse and 6.5 kb in human. Such large first introns are quite common in extracellular matrix genes

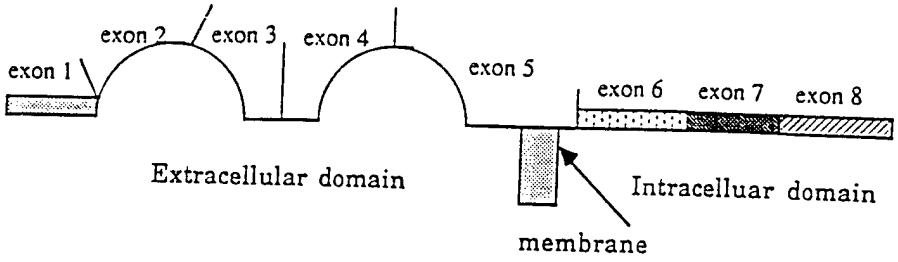

Fig. 4. Schematic diagram of EMMPRIN mRNA, showing exon borders and domain structures. Starting from the left is the 5′-UTR and the signal peptide encoded by exon 1. The two half circles indicate the Ig domains of the extracellular portion of the molecule, showing that each is encoded by two exons. The transmembranous domain is above the cross-hatched grey structure labeled 'membrane'. To the right of the transmembrane domain are the two exons encoding the cytoplasmic domain and one exon (exon 8) encoding most of the 3′-UTR.

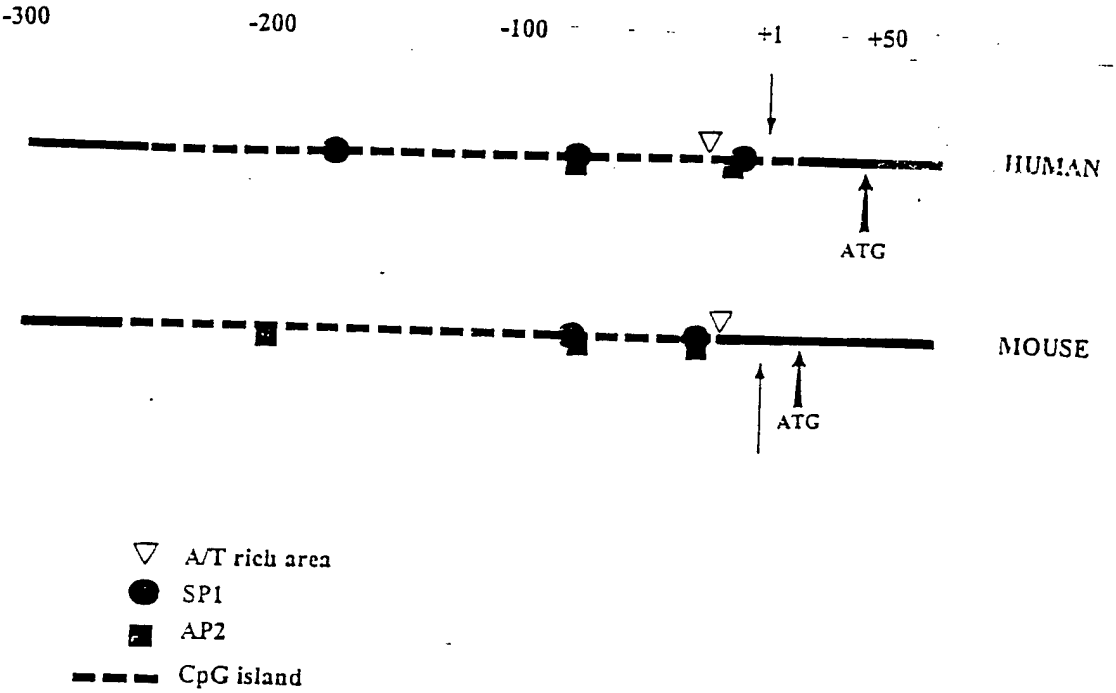

Fig. 5. Schematic of the 5′-flanking regions of the human and mouse EMMPRIN genes. The transcription start sites are aligned and are indicated by thin-based arrows below the −1 designation. The dashed line symbolizes the CpG island. Filled circles denote SP1 binding sites and filled boxes denote AP2 binding sites. The small A/T-rich regions that may facilitate RNA polymerase binding are indicated by open triangles. The ATG start codons are marked with thick-based arrows.

(Takahara et al., 1995; Truter et al., 1992; Yoshioka et al., 1995; Young et al., 1989) and may contain elements responsible for enhancing transcription or for conferring tissue-specific transcriptional regulation.

While the exon structure is highly conserved between human and mouse, some small species differences do exist. The human EMMPRIN gene contains eight exons, one more than the mouse gene. The equivalent of mouse exon 7 is encoded by exons 7 and 8 in the human gene. It is noteworthy that this region corresponds to the 3′-untranslated region of the mRNA. The significance of encoding the 3′-UTR in multiple exons is unknown. Another minor difference is observed at the exon 1/exon 2 border. The last amino acid encoded by human exon 1 is the first amino acid encoded by the mouse exon 2. The final minor difference involves the splitting of codons at the exon/intron borders. In the human gene, all exons begin and end with split codons. In the mouse this is true with the exception of the exon boundaries on both sides of intron 4, where complete 3 nucleotide codons are found.

The human gene, like the mouse gene (Cheng et al., 1994; Miyauchi et al., 1995), has the unusual property that each Ig domain is not encoded by one exon, but by two. Most members of the Ig superfamily encode the Ig domains in a single, unshared exon. Notable exceptions are some of the N-CAM Ig domains and CD4. Miyauchi and coworkers (Miyauchi et al., 1995) have suggested that this splitting of the Ig domains supports the idea that EMMPRIN is closely related to the primor-

dial polypeptide of the Ig superfamily, as also suggested from previously noted homologies with the IgV domain and β chain of MHC class II antigen (Miyauchi et al., 1990, 1991). Also unusual in the mouse and human genes compared with other Ig family members is the fact that the downstream exon of the second Ig domain is a junctional exon, encoding the transmembrane domain and part of the cytoplasmic domain as well. This, however, is a relatively common feature in modular extracellular matrix proteins. For example, junctional exons encoding a portion of a triple helical domain, as well as a portion of a non-collagenous domain, are customary in the collagen genes (Gordon et al., 1989; Sandell and Boyd, 1990; Tikka et al., 1988).

4.2. 5′-flanking region of the EMMPRIN gene

About 950 nucleotides of 5′-flanking sequence of the human EMMPRIN gene were determined and analyzed for consensus binding sites. We compared this region with the comparable region of the mouse basigin promoter, renumbering it to the style presented here for the human promoter. No consensus TATA or CAAT boxes were observed in either the human or mouse gene. An A/T-rich region that may allow access to RNA polymerase exists in the human gene at −21 to −28, and in the mouse gene at −15 to −22 (Fig. 5).

The mouse and human genes both contain consensus binding sites for the transcription factors SP1 and AP2, which are relatively common elements in extracellular matrix gene promoters. In the human sequence three SP1 sites are found (at approx. −175, −80 and −15). Two of these overlap with the two AP2 sites present (approx. −80 and −15). In the mouse three AP2 sites were found (approx. −200, −80 and −25); two of the three overlap the two SP1 sites present (approx. −80 and 25). The conservation of the overlapping AP2 and SP1 binding sites to approx. −80 and the general vicinity of approx. −20 in both species suggests that the position of these factors may be regulatory for the EMMPRIN gene.

The CpG island observed in the human gene between −247 and +6 is conserved in the mouse in an analogous position, −256 to −21. In housekeeping genes, these multiple CG dinucleotides are unmethylated and are thought to play a role in their constitutive expression. Interestingly, CpG islands also appear in the 5′-flanking regions of several extracellular matrix genes, which are not constitutively expressed (Bashir et al., 1989; Corson et al., 1993; Killen et al., 1988; Lee and Greenspan, 1995). The methylation state and the significance of the CpG island is unknown in matrix genes.

Matrix metalloproteinases are necessary for metastasis, yet most members of this enzyme family are produced by stromal cells associated with tumors rather than the tumor cells themselves (Heppner et al., 1996; Hewitt and Dano, 1996; MacDougall and Matrisian, 1995). Fibroblastic production of interstitial collagenase, stromelysin and 72 kDa gelatinase is stimulated by tumor cell-derived EMMPRIN (Guo et al., 1997; Kataoka et al., 1993), providing the most likely explanation for their expression in tumor stromal cells. The levels of expression of EMMPRIN in normal cells are low relative to tumor cells (DeCastro et al., 1996; Muraoka et al., 1993; Polette et al., 1997). Thus, it is likely that carcinogenic compounds or UV light indirectly trigger the up-regulation of the EMMPRIN gene, enhancing tumorigenesis. Elucidating the primary structure of the EMMPRIN promoter is our first step toward studying this process.

Acknowledgement

This work was supported by National Institutes of Health Grants, CA38817 (to C.B.) and EY09056 (to M.K.G.) and by Department of Defense Breast Cancer Research Grant, DAMD 17-95-1-5017 (to B.P.T.).

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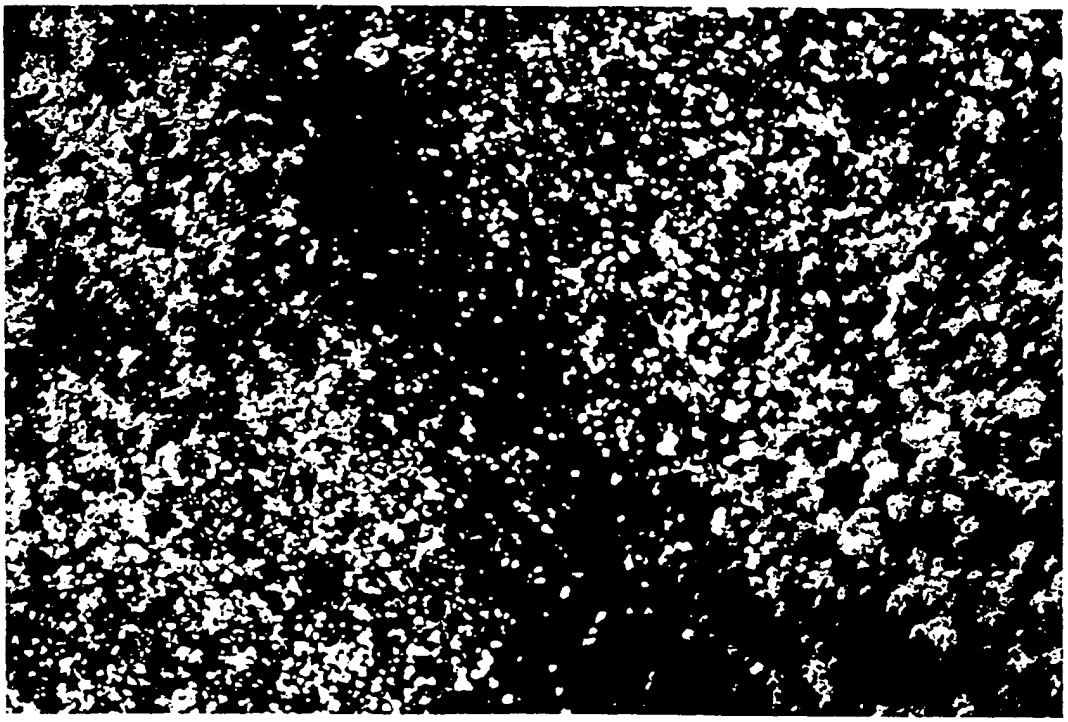

Published by Elsevier on behalf of the Federation of European Biochemical Societies

ISSN 0 014 5793

Volume 441

Number 1

11 December 1998

FEBLAL 441 (1) 1–158

FEBS Letters 441 (1998) 88–92 FEBS 21225

Tumor-derived EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates collagenase transcription through MAPK p38

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Received 25 October 1998

Abstract EMMPRIN (extracellular matrix metalloproteinase inducer) stimulates fibroblast metalloproteinases (MMP) 1, 2 and 3 (Kataoka et al. (1993) Cancer Res. 53, 3154–3158). Here we focus on MMP-1, showing that in lung tumors, MMP-1's cognate mRNA is strongly expressed in stromal fibroblasts adjacent to EMMPRIN-expressing tumor cells. In vitro, EMMPRIN upregulates MMP-1 mRNA expression in a concentration-dependent manner, with a peak accumulation at 24 h. The response is genistein-sensitive, suggesting it is dependent on tyrosine kinase activity. Analysis of tyrosine phosphorylation-dependent MAP kinases ERK 1/2, SAPK/JNK, and p38 showed that the activity of p38 but not that of the other 2 kinases was elevated in response to EMMPRIN. That p38 activity was required for EMMPRIN stimulation of MMP-1 was evident from results showing that the p38 inhibitor SB203580 blocked this response. This is the first available information regarding the mechanism by which tumor-associated molecules upregulate MMP synthesis in stromal fibroblasts.

Key words: Extracellular matrix metalloproteinase inducer; Metalloproteinase; MAP kinase; p38; Matrix metalloproteinase-1

1. Introduction

Tumor growth and metastasis require the breakdown of extracellular matrix, the components of which may be broken down collectively by matrix metalloproteinases. The regulation of these enzymes in tumorigenesis is poorly understood [1–3]. Although both tumor and stromal cells express MMPs localization studies show that fibroblasts are the primary source of MMPs in most tumors [4–8]. Our survey of human non-small cell lung carcinomas was consistent with this, thus supporting the notion that tumor-derived factors stimulate nthesize MMPs.

The purpose of the present study was to identify elements of the signaling pathway mediating extracellular matrix metalloproteinase inducer (EMMPRIN) stimulation of fibroblast MMP-1. Using chemical inhibitors of key signaling molecules, we determined that protein tyrosine kinases play an important role. More specifically, the activity of the p38 MAP kinase was required for the response. This is the first information regarding the mechanism by which tumor cells activate MMP-1 in adjacent fibroblasts, and demonstrates that p38 MAP kinase is an important regulator of MMP-1 gene expression.

2. Materials and methods

2.1. Materials

Samples of non-small cell lung carcinomas were collected at UCSF-affiliated hospitals (Moffitt-Long Hospital, UCSF/Mount Zion Hospital, and the Veterans Administration Hospital). Samples were frozen with or without fixation in 10% formalin. 16-Lu lung fibroblasts were purchased from the American Tissue Cell Culture facility (Bethesda, MD, USA). T7 and SP6 RNA polymerases, salmon sperm DNA, *Eco*RI restriction enzyme, random priming kits, and Trizol reagent were from Gibco. Probes used in Northern blots were produced by PCR using human cDNA as template. PCR products were cloned into TA cloning vector (Invitrogen). Acrylamide, Trizma base, boric acid, NaCl, tRNA, and sodium citrate were from Sigma. Ilford K5D emulsion was purchased from Polysciences. Kodak developer and fixative were used for in situ hybridization. Genistein, bisindolylmaleimide, PD98059, PP-1, KT5720, pertussis toxin, and SB203580 signal transduction inhibitors were from Calbiochem (La Jolla, CA, USA). Antibodies against p42/44, p38, and SAPK/JNK were purchased from New England Biolabs (Beverly, MA, USA).

2.2. Purification of EMMPRIN

EMMPRIN was purified from non-small cell lung carcinoma cell (either LX-1 or NCI H460) membranes. Homogenates from these cells were passed over immunoaffinity columns made with monoclonal [9] or polyclonal anti-EMMPRIN antibody (made with Affi-Gel 10, Bio-Rad). Rabbit anti-EMMPRIN polyclonal antiserum was generated against the synthetic peptide NH_2-DALPGQKTEEKVDSDDQW-GC-COOH [10]. The antibody recognized purified native EMMPRIN on Western blot (data not shown). For purification, lung tumor cell homogenates were loaded onto the column and the column was washed several times with 0.1 M HEPES buffer. EMMPRIN was eluted with 0.1 N acetic acid and concentrated in Centriprep and Centricon-10 columns (Amicon; Beverly, MA, USA).

2.3. In situ hybridization

Frozen sections of tumor and normal lung were probed with sense and antisense ^{35}S-labeled ribonucleoprobes corresponding to EMMPRIN, MMP-1 and MMP-2, and in situ hybridization was carried out as described [11]. Briefly, 6-μm thick tissue sections were fixed in 4% paraformaldehyde and treated with proteinase K and acetic anhydride prior to hybridization. The slides were incubated overnight at 55°C, then washed with 0.1×SSC at 62°C for 2 h. After dehydration they were dipped in emulsion and exposed for 2–3 weeks prior to developing. EMMPRIN, MMP-1 and MMP-2 were localized with riboprobes synthesized from nucleotides 73–403, 825–1091, and 1409–1747, respectively, of their cognate cDNAs [10,12,13]. All probes were cloned into pTA3 cloning vector [11].

2.4. Cell culture and Northern blot

Human lung fibroblasts (16-Lu and 13-Lu) and human fetal colon fibroblasts (CCD-18) were grown in Dulbecco's modified Eagle's

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PII: S0014-5793(98)01474-4

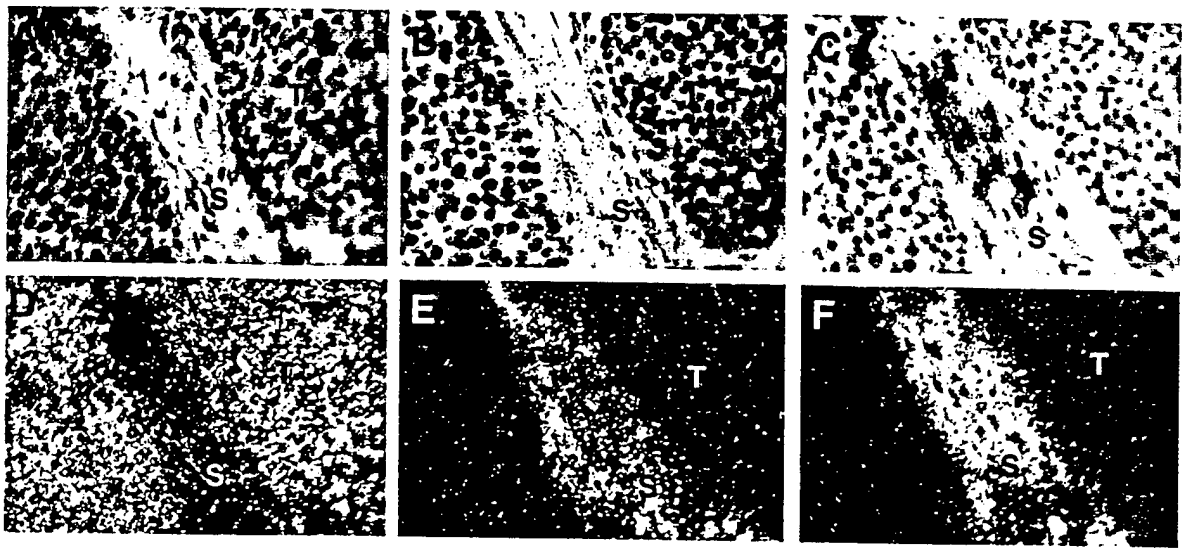

Fig. 1. In situ hybridization of NSCLC, brightfield and darkfield views. EMMPRIN probe (A, D) is strongly localized to tumor (T), whereas both MMP-2 (B, E) and MMP-1 (C, F) mRNAs are localized to stromal cells (S) between tumor islands. Magnification 100×.

(DME) medium/F12 (50:50) with essential amino acids (Gibco), supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were grown to confluence in 6-well plates prior to starvation in serum-free medium. After 30 h the cells were treated with EMMPRIN or vehicle control. RNA was collected with Trizol reagent according to the manufacturer's instructions.

Total RNA (5–10 μg) was separated on a 1% agarose/formaldehyde gel and transferred to nylon (GeneScreen) in 10× SSC buffer. The RNA was fixed by UV crosslinking (Stratalinker 1800, Stratagene, Cambridge, MA, USA). The MMP-1 probe was excised from TA cloning vector with *Eco*RI and labeled with ^{32}P-dCTP by random priming. The GAPDH cDNA probe was made as described [11] and used as an internal control. Hybridization was performed with ExpressHyb (Clonetics). After 1 h the membranes were washed with 1× SSC at room temperature for 10 min 2× and 1× SSC/1% SDS at 50°C for 30 min. The membranes were exposed overnight at −80°C before developing. Quantification of data was by densitometry.

2.5. Stimulation of fibroblasts with purified EMMPRIN

16-Lu, CCD-18, and 13-Lu fibroblasts were stimulated with purified EMMPRIN (1–3 μg/ml) for 24 h prior to RNA extraction. In time course experiments total RNA from 16-Lu cells was extracted after 2, 6, 12, 24, and 48 h. To assess concentration-dependence, one tenth of the dose (0.3 μg) used in these experiments was added to 16-Lu fibroblasts and RNA was collected at 24 h.

2.6. Inhibition of EMMPRIN stimulation by signal transduction inhibitors

16-Lu fibroblasts were stimulated with EMMPRIN in the presence of various signal transduction inhibitors used at saturating concentration [14–17]. Genistein (67 μM), bisindolylmaleimide I (10 nM), PD98059 (10–100 μM), PP-1 (30 μM), KT5720 (0.5 μM), pertussis toxin (100 ng/ml), and SB203580 (1–100 μM) were added to cells in serum-free medium one hour before and for the duration of EMMPRIN exposure. RNA was collected after 12 h and analyzed for the presence of MMP-1 and GAPDH RNA.

2.7. Immunoblotting analysis of Erk1/2, p38, and SAPK/JNK MAP kinases

16-Lu fibroblasts were stimulated with EMMPRIN for 0, 15, 30, and 60 min prior to harvesting of total cellular protein. For each time point equal amounts of protein were electrophoresed under reducing conditions. Identical gels were run simultaneously and the proteins then transferred to PVDF membrane (Immobilon-P, Millipore, Bed-

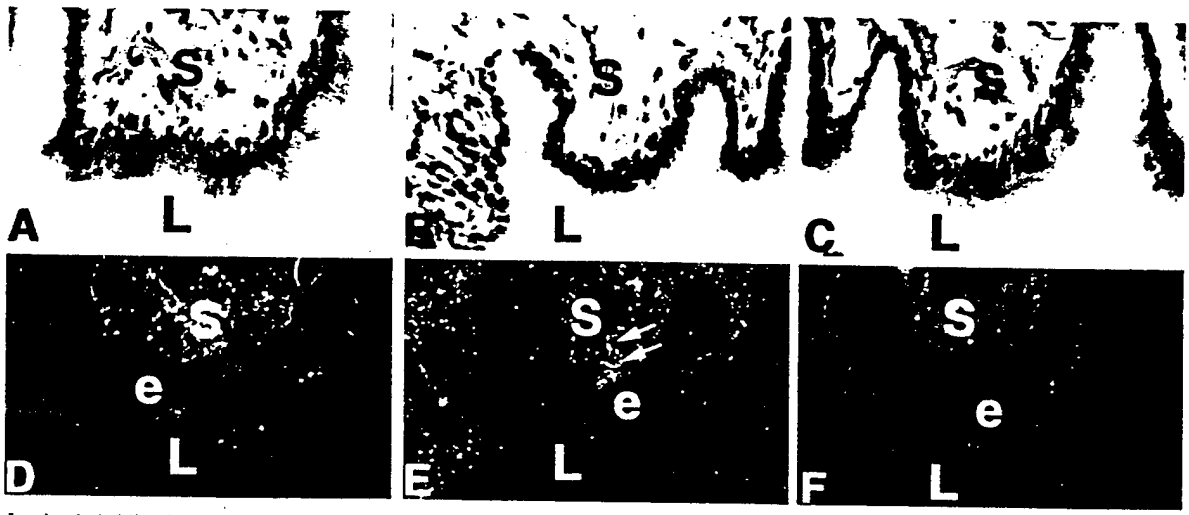

Fig. 2. In situ hybridization of normal bronchial epithelium, brightfield and darkfield views. EMMPRIN (A, D) and MMP-1 (C, F) probes reveal little to no signal, whereas MMP-2 (B, E) shows low intensity signal in subepithelial fibroblasts (white arrows in E). S = stroma, L = lumen, e = epithelium. Magnification 100×.

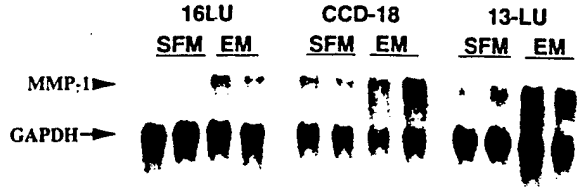

Fig. 3. EMMPRIN upregulation of MMP-1 mRNA in three fibroblast cell lines. With each shown in duplicate, EMMPRIN (EM) or serum-free medium (SFM) was administered to 16-Lu, CCD-18, and 13-Lu fibroblasts for 24 h, then total RNA extracted for Northern blotting. EMMPRIN upregulates steady state MMP-1 mRNA in all 3 cell lines. GAPDH is shown as an internal control.

ford, MA, USA). After blocking with 5% (w/v) non-fat dry milk, the blots were incubated for one hour with antibodies directed against phosphorylated and non-phosphorylated forms of Erk/p44/42, SAPK/JNK, and p38. Blots were washed 5 min 3× with Tris-buffered saline/0.1% Tween-20 and incubated with horseradish peroxidase-conjugated secondary antibody (1:2000) for one hour. The membranes were washed as above, and the bands detected by chemiluminescence after 30 s–1 min exposures.

3. Results

3.1. EMMPRIN mRNA is in tumor cells adjacent to MMP-1 and -2 expressing fibroblasts

Rapidly frozen NSCLC (6 samples) and normal lung (3 samples) specimens were examined for RNA expression of EMMPRIN, MMP-1, and MMP-2. In both squamous cell ($n=4$) and adenocarcinoma ($n=2$) histologic subtypes, carcinoma cells uniformly expressed high levels of EMMPRIN (Fig. 1A and D) and adjacent stromal fibroblasts strongly expressed MMP-1 and MMP-2 (Fig. 1B, C, E, F). The latter is consistent with previous studies reporting elevated metalloproteinase expression in lung and head/neck tumors [18–20].

Normal lung samples did not contain detectable levels of EMMPRIN and MMP-1 mRNAs (Fig. 2A, D, C, F). MMP-2 was weakly expressed in subepithelial fibroblasts (Fig. 2B and E).

3.2. EMMPRIN stimulates MMP-1 steady state mRNA in multiple fibroblast cell lines

Fibroblast cell lines from both adult lung (16-Lu and 13-Lu) and fetal colon (CCD-18) showed increased MMP-1 RNA in response to EMMPRIN (Fig. 3), although the degree of stimulation differed.

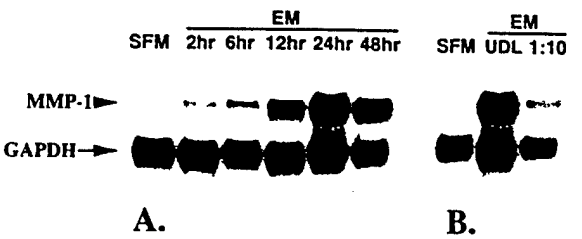

Fig. 4. Time course and concentration dependence of MMP-1 upregulation. A: EMMPRIN (1–3 μg/ml) was administered to 16-Lu fibroblasts for 2, 6, 12, 24, or 48 h, then total RNA collected. MMP-1 steady state mRNA begins to rise at 2 h and peaks at 24 h (B). EMMPRIN was administered at 3 μg/ml (undiluted, UDL) or 0.3 μg/ml (1:10) for 24 h before RNA collection. Effect on MMP-1 mRNA is concentration-dependent.

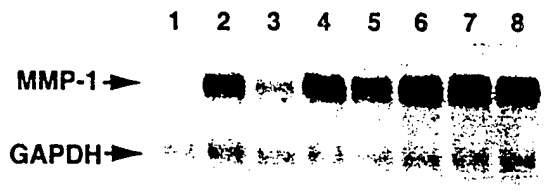

Fig. 5. Effect of signal transduction inhibitors on EMMPRIN stimulation of MMP-1. EMMPRIN (3 μg/ml) was co-administered with various inhibitors for 10 h prior to total RNA collection. 1: Serum-free medium; 2: EMMPRIN alone; 3–8: EMMPRIN plus (3) genistein, (4) bisindolylmaleimide, (5) PP-1, (6) PD98059, (7) KT5720, (8) pertussis toxin. Only genistein shows reduction in MMP-1 steady state mRNA.

3.3. EMMPRIN stimulation of MMP-1 mRNA peaks at 24 h and is dose dependent

A kinetic analysis of EMMPRIN-induced MMP-1 mRNA showed a peak intensity of MMP-1 steady state mRNA at 24 h (Fig. 4A). The response at 24 h was concentration-dependent (Fig. 4B).

3.4. EMMPRIN activates protein tyrosine kinases

A survey of the effects of various signal transduction inhibitors showed a sensitivity of EMMPRIN signaling to the protein tyrosine kinase inhibitor genistein (Fig. 5). In contrast, bisindolylmaleimide (PKC inhibitor), PD98059 (MEK1/2 inhibitor), KT5720 (PKA inhibitor), PP-1 (Src kinase inhibitor), and pertussis toxin (G protein inhibitor) did not inhibit the response. Because protein tyrosine kinases play an important

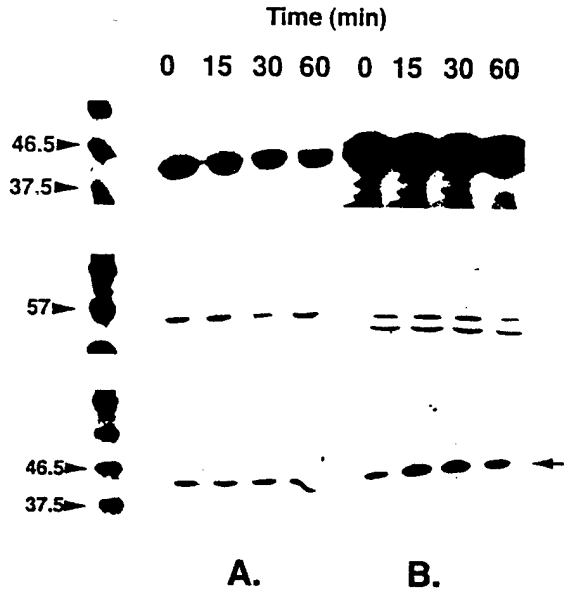

Fig. 6. ERK, SAPK/JNK, and p38 immunoblotting. Total cellular protein from 16-Lu cells stimulated with EMMPRIN (3 μg/ml) for 0, 15, 30 and 60 min was analyzed by 10% SDS-PAGE and transferred to membrane prior to immunoblotting. Reactions with antibodies directed against the non-phosphorylated (A) and phosphorylated (B) forms of 3 MAP kinases are shown. Phosphorylation of p38 at 15 min (lower panel, B) is detected. ERK1/2 (upper panel, B) and JNK/SAPK (middle panel, B) do not show increased phosphorylation. Reactions with antibodies detecting non-phosphorylated MAP kinases (upper, middle, and lower panels, A) are included as controls. Biotinylated markers are on the left of each panel.

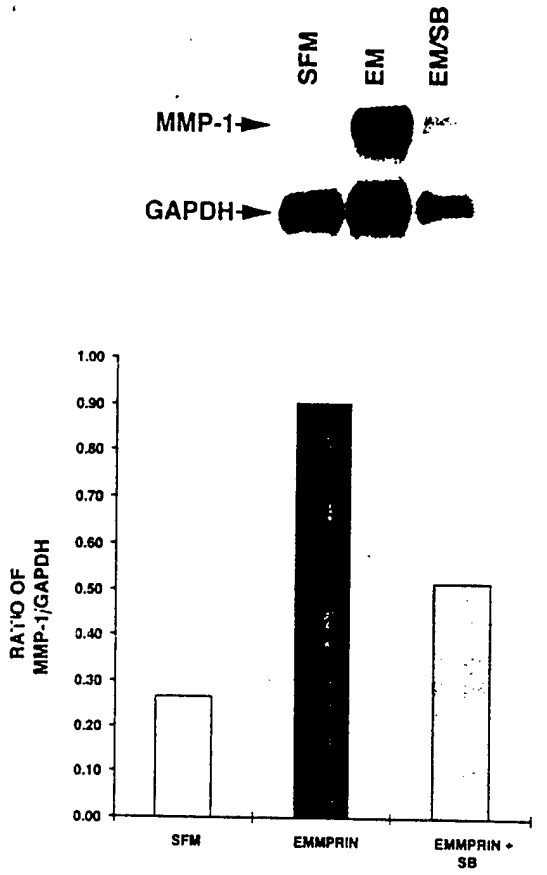

Fig. 7. SB203580 inhibits EMMPRIN upregulation of MMP-1. The specific p38 inhibitor, SB2035580 (10 μM), was preadministered 1 h and co-administered with EMMPRIN for 10 h prior to total RNA collection. The graph compares MMP-1/GAPDH signal intensity and indicates a 45% inhibition by SB203580. SFM = serum-free medium: EM = EMMPRIN alone: EM/SB = EMMPRIN with SB203580.

role in MAP kinase activation, we investigated the potential role of MAP kinases.

3.5. *EMMPRIN stimulates phosphorylation of p38 but not other MAP kinases*

EMMPRIN stimulated phosphorylation of p38 but not Erk1/2 or JNK (Fig. 6). Anti-phospho-p38, which detects dual phosphorylation of p38 at Tyr-182 and Thr-180, showed enhancement after 15- and 30-min exposure to EMMPRIN, declining at 60 min (Fig. 6, lower panel). No increased signal was detected with the phospho-Erk/p44/p42 or phospho-SAPK/JNK antibodies (Fig. 6, upper and middle panels).

3.6. *SB203580 inhibits EMMPRIN upregulation of MMP-1 mRNA*

To determine whether the observed stimulation of p38 is required for EMMPRIN upregulation of MMP-1, we tested the effect on the response of the p38 inhibitor SB203580. When SB203580 was co-administered (at saturating doses) with EMMPRIN, the response decreased by ~45% (Fig. 7A and B).

4. Discussion

Our results show that the tumor-associated protein EMMPRIN stimulates metalloproteinase-1 mRNA in fibroblasts via a p38-dependent signaling pathway. These results are important in light of the fact that metalloproteinase activity has been shown to be necessary for tumor growth [21]. It seems likely that signaling mechanisms by which tumors increase local metalloproteinase activity may constitute novel anti-tumor drug targets.

MMP-1 is a member of the matrix metalloproteinase family, zinc-dependent metalloenzymes that contribute importantly to both normal tissue remodeling [22–25] and pathological processes [26–29]. As a type I collagenase, it has the unique ability to cleave fibrillar collagen [2]. Although synthesis of metalloproteinases shows a general, albeit differential, sensitivity to growth factors and cytokines [30,31], and specific tyrosine kinase- [14], protein kinase C- [14], protein kinase A- [32], and Src- [33] dependent mechanisms have been identified, the control of these enzymes by extracellular stimuli is largely unknown.

We focused on the control of MMPs by the tumor-associated protein EMMPRIN. Results of a pharmacological screen showed that tyrosine kinases, but not PKA, PKC, or Src are required for EMMPRIN induction of MMP-1. Since tyrosine kinases are integrally involved in MAP kinase signaling pathways, we subsequently examined the effects of inhibitors of these pathways, specifically the MEK1/2 inhibitor PD98059 and the p38 inhibitor SB203580. The latter significantly attenuated the response to EMMPRIN, indicating a role for p38 activation in the induction of MMP-1. Consistent with this, p38 was the only one of three major MAP kinases (ERK1/2, SAPK/JNK, and p38) that underwent phosphorylation (an index of activation) in response to EMMPRIN. At saturating doses of the p38 inhibitor, MMP-1 induction was decreased by only ~45%. Clearly, although p38 plays an important role in EMMPRIN induction of MMP-1, it does not account entirely for its upregulation. Potential contributors to this induction may include less well-described MAP kinase pathways [34] or posttranscriptional mechanisms (e.g. prolongation of RNA half life).

Interestingly, p38 has recently been implicated in the induction of another metalloproteinase, MMP-9, by phorbol ester [15]. Originally described by Han et al. [35], p38 is generally regarded as a stress-activated enzyme and has been shown to have downstream effects on transcription factor activation [36], actin filament rearrangement [37], and matrix degradation ([15] and this report). As such, p38 eventually may be recognized to play a variety of important roles in the general process of tissue remodeling.

The identity of signaling molecules linking p38 and MMP-1 transcription remains unknown. Clues are provided, however, by information regarding known response elements on the MMP-1 promoter. There is a well-described AP-1 binding site at −72/−67 [38] that is responsive to a variety of stimuli including phorbol esters, TGF-β, cAMP and TNF-α [39,40]. AP-1 is activated by a variety of signaling cascades, some of which are p38-dependent [36]. Studies to determine the locus of the EMMPRIN response element associated with the MMP-1 gene are in progress.

Acknowledgements: We thank Wilfred Raymond and George Caughey for their assistance with EMMPRIN purification, and Marianne Gallup for help with the preparation of figures. This work was supported by grants P01 HL43762 (CB) and R01 HL24136 (CB).

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[CANCER RESEARCH 60, 888–891, February 15, 2000]

Advances in Brief

EMMPRIN (CD147), an Inducer of Matrix Metalloproteinase Synthesis, Also Binds Interstitial Collagenase to the Tumor Cell Surface[1]

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Abstract

Extracellular matrix metalloproteinase inducer (EMMPRIN), also known as basigin or CD147, is a glycoprotein that is enriched on the surface of tumor cells and stimulates production of several matrix metalloproteinases by adjacent stromal cells. In this study, we have found that EMMPRIN not only stimulates the production of interstitial collagenase (MMP-1) but also forms a complex with MMP-1 at the tumor cell surface. Complex formation was demonstrated by phage display, affinity chromatography, and immunocytochemistry. Presentation of MMP-1 complexed to EMMPRIN at the tumor cell surface may be important in modifying the tumor cell pericellular matrix to promote invasion.

Introduction

MMPs[3] have been implicated in several aspects of tumor progression, including invasion through basement membranes and interstitial matrices, angiogenesis, and tumor cell growth (1–3). Strong support for the involvement of MMPs at some step in tumor progression comes from experiments in which tissue inhibitors of MMPs or synthetic inhibitors of metalloproteinases have been shown to reduce tumor growth and metastasis (4, 5). Over the past several years, it has become increasingly apparent that tumor cells create a pericellular environment in which MMPs and other proteases become concentrated, thus enhancing the ability of tumor cells to invade extracellular matrices (6–8). Previous studies from this laboratory have demonstrated that EMMPRIN, a member of the immunoglobulin superfamily that is enriched on the surface of most tumor cells, stimulates stromal cells to produce elevated levels of several MMPs, including MMP-1 (9–11). We have now found that tumor cell EMMPRIN not only stimulates MMP-1 production by fibroblasts but also binds MMP-1 to the surface of tumor cells, thus adding to the complement of proteases on the tumor cell surface that may promote invasion.

Materials and Methods

Phage Display Library. mRNA was prepared from human fibroblasts with the Oligotex mRNA kit (Qiagen, Valencia, CA) and used for cDNA synthesis with the Directional RH primer cDNA synthesis kit (Novagen, Madison, WI). After second-strand synthesis, the cDNA ends were flushed with T4 DNA polymerase and ligated to *Eco*RI/*Hin*dIII directional linkers. The cDNA was then digested with *Eco*RI and *Hin*dIII and ligated to T7Select1-1b vector arms (Novagen). The ligated DNA was packaged into bacteriophage T7 using the T7Select1-1 Packaging Extract (Novagen). The host strain of bacteria, BLT 5403 (Novagen), was then grown to $A_{600\ nm}$ = 0.8–1.0 and mixed with the packaged cDNA (at a ratio of 10^6 phage/10 ml cells) in LB media containing 50 μg/ml carbenicillin (Novagen). Molten top agarose at 45°C–50°C was added to the phage/host mixture (10:1) and immediately poured onto a 150-mm plate containing LB/carbenicillin medium. The plate was incubated at room temperature overnight until the plaques were nearly confluent. The phage was then eluted by covering the plate with phage extraction buffer [100 mM NaCl, 20 mM Tris, and 6 mM $MgSO_4$ (pH 8.0)] at 4°C overnight. The phage lysate was clarified with chloroform and subjected to screening by biopanning.

Screening of Phage Display Library. Twenty four-well cell culture plates were prepared for biopanning as suggested by the manufacturer (Novagen). The wells were coated with immunopurified EMMPRIN protein (Ref. 12; 1 μg/ml in Tris-buffered saline) at 4°C overnight and washed with Tris-buffered saline five times. Unreacted sites were blocked with 5% blocking reagent overnight at 4°C and washed. In the first round of screening, the phage lysate was applied to the EMMPRIN-coated plate (0.5 ml lysate/well) for 30 min at room temperature. The plate was then washed five times with Tris-buffered saline. The bound phages were eluted by adding 0.5 ml of elution buffer (1% SDS) at room temperature for 20 min. The eluted phages were then added to a culture of the host cells (BLT 5403) in LB media and incubated at 37°C with shaking for 3 h, at which time lysis was observed. The lysed culture was centrifuged, and the supernatant was collected for the next round of biopanning. A total of five rounds of screening was carried out. DNA from the phages isolated during the final round of screening was purified and sequenced using the T7 SelectUp primer (GGAGCTGTCGTATTCCAGTC) and the T7 SelectDown primer (AACCCTCAAGACCCGTTTA; Novagen).

Immunoaffinity and Ligand Affinity Chromatography. EMMPRIN was isolated from extracts of membranes from LX-1 human lung carcinoma cells by immunoaffinity chromatography using E11F4 monoclonal antibody against EMMPRIN immobilized on Sepharose beads, as described previously (12).

For manufacture of the ligand affinity medium, EMMPRIN protein (0.5 mg) was first dissolved in coupling buffer [0.1 M $NaHCO_3$ and 0.5 M NaCl (pH 8.3) containing 0.5% NP40]. The coupling solution was then mixed with CNBr-activated Sepharose 4B gel (Pierce; 0.25 g of dried powder swelled and washed in 1 mM HCl for 30 min) at 4°C. After overnight incubation, the gel was washed three times with 5 ml of coupling buffer, followed by incubation in 0.1 M Tris-HCl (pH 8) for 2 h to block any remaining active groups. Then the gel was washed using three cycles of 0.1 M acetate buffer, 0.5 M NaCl (pH 4), and 0.1 M Tris and 0.5 M NaCl (pH 8). After washing, the gel was resuspended in 5 ml of 10 mM Tris buffer (pH 8.3).

Extracts of human fibroblasts [10^8 cells in 5 ml of 10 mM Tris, 0.15 M NaCl, and 0.5% NP40 (pH 8.3)] were added to the EMMPRIN-coupled gel and incubated at 4°C overnight with rotation. The gel was then washed with 10 mM Tris and 0.15 M NaCl containing 30 mM octyl glucoside until the $A_{280\ nm}$ was less than 0.05. Binding proteins were eluted with 0.1 M glycine buffer (pH 2.5) containing 30 mM octyl glucoside. The eluate was neutralized to pH 7 by the addition of 1 M Tris (pH 9.5) and concentrated for further analysis.

ELISA of MMP-1. MMP-1 protein was measured in the eluates from EMMPRIN-Sepharose and in immunopurified EMMPRIN preparations using a commercial ELISA system (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Briefly, 5 or 10 μl of eluate were added to microtiter plates coated with antibody to MMP-1 and incubated for 2 h at 25°C. The plates were washed with phosphate buffer and incubated with anti-MMP-1 antiserum for 2 h. After washing, the plates were incubated with peroxidase-conjugated secondary antibody for 1 h, and processed for color development and measurement at $A_{450\ nm}$ in a microplate spectrophotometer. The concentration of MMP-1 in the eluate was estimated from a standard curve.

Received 11/18/99; accepted 1/3/00.

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[1] Supported by United States Army Grants DAMD17-95-1-5017 and DAMD17-99-9413.

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[3] The abbreviations used are: MMP, matrix metalloproteinase; EMMPRIN, extracellular matrix metalloproteinase inducer; MMP-1, interstitial collagenase; MMP-2, gelatinase A; MT-MMP, membrane-type MMP; LB, Luria-Bertani.

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SDS-PAGE, Silver Staining, and Western Blotting. Proteins were dissolved in SDS sample buffer containing 0.1 M DTT and heated at 95°C for 5 min. The samples were then subjected to electrophoresis on 10% SDS polyacrylamide gels. The gels were either stained using the Sterling silver staining system (National Diagnostics, Atlanta, CA) or electroblotted onto nitrocellulose membranes and incubated with antibody against EMMPRIN (E11F4; Ref. 12) or against MMP-1 (Calbiochem, La Jolla, CA) for 1 h at room temperature. The immunoreactive protein bands were detected with horseradish peroxidase-conjugated antimouse IgG and chemiluminescence reagent (New England Nuclear Life Science, Boston, MA).

Immunocytochemistry. LX-1 human lung carcinoma cells were seeded into chamber culture slides and cultured for 48 h at 37°C in 5% CO_2 air. The cells were then washed with PBS, fixed in 1% paraformaldehyde in PBS for 45 min at room temperature, quenched with 0.1 M Tris (pH 7.4), and blocked with 1% BSA, 1% goat serum, and 2% nonfat milk in PBS at room temperature for 1 h. The LX-1 cells were then incubated with monoclonal antibody against MMP-1 (Calbiochem) for 1 h at room temperature, followed by Cy3-conjugated Texas red goat antimouse IgG. The cells were washed with PBS, mounted with coverslips, and then observed and photographed using a Zeiss Axioskop-20 microscope.

Results

Phage Display Reveals MMP-1 as an EMMPRIN-binding Protein. We used the T7Select Phage Display System (Novagen) to identify EMMPRIN-binding protein(s) encoded by a cDNA library prepared from human fibroblasts, as described in "Materials and Methods." In this method, each phage becomes coated with a fusion protein comprised of the phage coat protein and a protein generated from the cDNA library used. Phages coated with putative EMMPRIN-binding protein were selected by repeated panning over 24-well plates coated with EMMPRIN. Five rounds of biopanning were carried out, and the final lysate was used for plaque assay, PCR amplification, and sequencing.

Eight clones were obtained from the procedure described above. All eight of the inserts were of identical size, *i.e.*, 0.8 kb, and were found to have identical sequences corresponding exactly to a portion of the human MMP-1 sequence (Fig. 1).

MMP-1 Binds to EMMPRIN-Sepharose. To confirm the binding of EMMPRIN to fibroblast-produced MMP-1, we performed ligand chromatography over Sepharose conjugated with immunopurified EMMPRIN. Fibroblast extracts were mixed with the EMMPRIN-Sepharose, which was then washed and eluted as described in "Materials and Methods." The eluates were subjected to SDS-PAGE, followed by silver staining. On silver staining, a prominent protein band at $\sim M_r$ 55,000 was observed, as well as a weaker band at $\sim M_r$ 67,000 (Fig. 2*A*); in some cases a $\sim M_r$ 45,000 band could also be seen.

Western blots were also performed on the eluates from EMMPRIN-Sepharose using antibody against human MMP-1. The protein band at $\sim M_r$ 55,000 (the approximate size of pro-MMP-1, which is M_r 52,000) reacted with anti-MMP-1 antibody (Fig. 2*B*), confirming our results from the phage display. ELISA measurements also revealed MMP-1 in the eluates from EMMPRIN-Sepharose (data not shown). The identities of the $\sim M_r$ 67,000 and $\sim M_r$ 45,000 proteins are not yet known.

EMMPRIN Forms a Complex with MMP-1 on the Surface of Tumor Cells. Some tumor cells themselves produce small amounts of MMP-1. Thus, we also determined whether, in addition to binding isolated EMMPRIN protein, MMP-1 forms a complex with EMMPRIN present on the surface of LX-1 human lung carcinoma cells. We immunopurified EMMPRIN from extracts of LX-1 cell membranes using monoclonal antibody E11F4 covalently bound to Sepharose beads and tested whether MMP-1 was present in the eluted EMMPRIN preparation. Fig. 3 shows a Western blot of such an

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                1                                                          55
5' sequence:    GGGAGAAGTGATGTTCTTTAAAGACAGATTCTACATGCGCACAAATCCCTTCTAC
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human MMP-1:    GGGAGAAGTGATGTTCTTTAAAGACAGATTCTACATGCGCACAAATCCCTTCTAC
                941                                                        995

                56                                                         110
                CCGGAAGTTGAGCTCAATTTCATTTCTGTTTTCTGGCCACAACTGCCAAATGGGC
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                CCGGAAGTTGAGCTCAATTTCATTTCTGTTTTCTGGCCACAACTGCCAAATGGGC
                996                                                       1050

                111                                                        165
                TTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTTTTCAAAGGGAA
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                TTGAAGCTGCTTACGAATTTGCCGACAGAGATGAAGTCCGGTTTTTCAAAGGGAA
                1051                                                      1105

                1                                                          55
3' sequence:    GGAGTGCCTGATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGC
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Human MMP-1:    GGAGTGCCTGATGTGGCTCAGTTTGTCCTCACTGAGGGGAACCCTCGCTGGGAGC
                343                                                        397

                56                                                         110
                AAACACATCTGACCTACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGA
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
                AAACACATCTGACCTACAGGATTGAAAATTACACGCCAGATTTGCCAAGAGCAGA
                398                                                        452

                111                131
                TGTGGACCATGCCATTGAGAA
                |||||||||||||||||||
                TGTGGACCATGCCATTGAGAA
                453                473
```
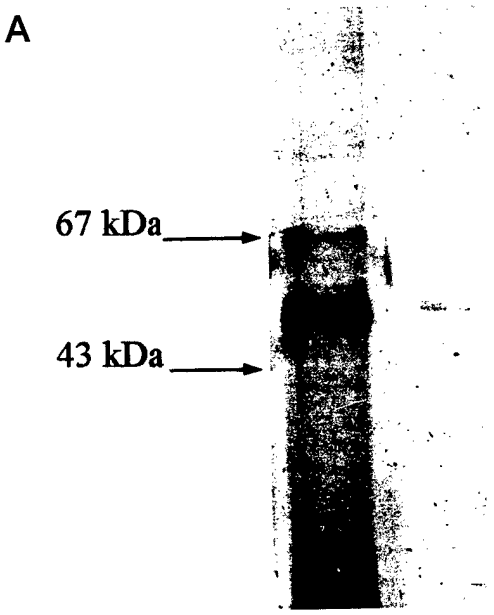

Fig. 1. Comparison of partial sequences of cDNA isolated by phage display with that of human MMP-1 cDNA. The 5′ and 3′ sequences of one of the partial cDNAs obtained are given. Eight cDNA clones were isolated after biopanning of phages on EMMPRIN; all eight clones had identical sequences.



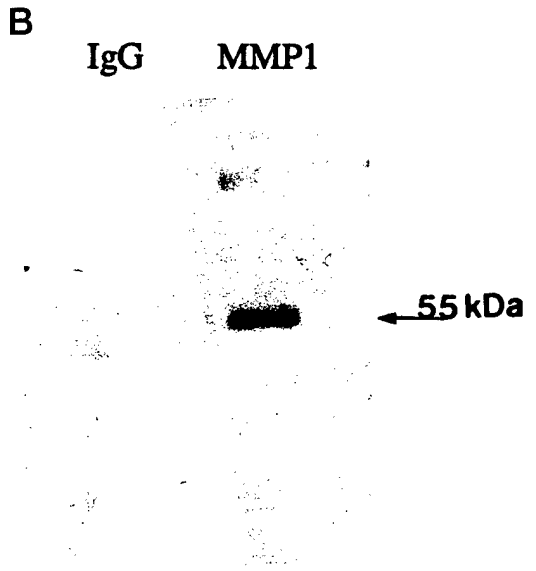


Fig. 2. EMMPRIN affinity chromatography of proteins extracted from human fibroblasts. *A*, proteins recovered from chromatography of fibroblast extracts on EMMPRIN-Sepharose were run on SDS-PAGE and silver-stained; two bands (at ~M_r 55,000 and ~M_r 67,000) were detected. *Arrows* indicate positions of the M_r 43,000 and M_r 67,000 markers. *B*, parallel gels to those in *A* were transblotted and reacted with antibody to MMP-1 or secondary antibody only (*IgG*); the ~M_r 55,000 band reacted with anti-MMP-1.

EMMPRIN preparation with antibody against MMP-1. A strong band at ~M_r 55,000, corresponding approximately in size to pro-MMP-1, reacted with the antibody, indicating the presence of MMP-1 in the EMMPRIN preparation. A weaker band at ~M_r 45,000, which is not seen consistently, is most likely activated MMP-1 (M_r 42,000).

Quantitation of the MMP-1 content by ELISA gave 2.1 μg of MMP-1 per 5 μg of total protein in the EMMPRIN preparation. Because EMMPRIN and pro-MMP-1 have molecular weights of ~58,000 and 52,000, respectively, this result suggests that EMMPRIN and MMP-1 are complexed in an equimolar ratio.

The presence of MMP-1 at the surface of LX-1 human lung carcinoma cells was confirmed by immunocytochemistry using antibody against MMP-1 (Fig. 4).

Discussion

Many recent studies have highlighted the importance of the pericellular milieu surrounding tumor cells in their proliferative and invasive behavior (6–8). This milieu is modified by a number of proteases, especially MMPs and tissue serine proteases, many of which are produced by tumor-associated stromal cells rather than tumor cells themselves (13, 14) and subsequently become concentrated at the tumor cell surface via interaction with specific binding

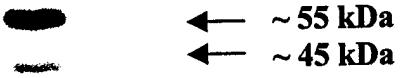


Fig. 3. Western blot of immunopurified EMMPRIN with antibody to MMP-1.

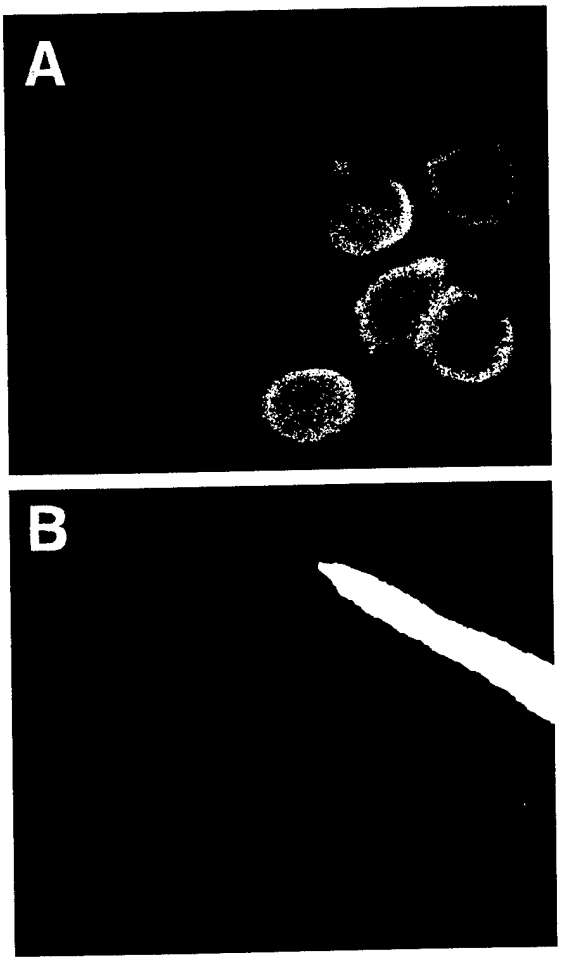


Fig. 4. Immunoreactivity of LX-1 human carcinoma cells with antibody to MMP-1. *A*, cells stained with antibody against MMP-1. *B*, cells stained with secondary antibody only.

sites. For example, MMP-2 binds to the tumor cell surface via a tissue inhibitor of MMPs-2-MT-MMP complex (15, 16). MMP-2 is activated by the MT-MMP, and the complex is targeted to invasive domains of the tumor cell membrane (sometimes termed "invadopodia") via specific docking of MT-MMP at these sites (17). Although MT-MMPs activate soluble MMP-2 as well as plasma membrane-retained MMP-2, membrane-bound enzyme is required for tumor cell invasion (17). A similar mechanism of activation and retention at the cell surface has been described for collagenase 3 (18). Other cell surface binding sites have been described for gelatinase B, *i.e.*, CD44 (19) and the α_2(IV) chain of collagen (20), and for MMP-2, *i.e.*, $\alpha_v\beta_3$ integrin (21). These sites also appear to be important in tumor cell invasion.

Evidence for association of MMP-1 with the surface of a human pancreatic carcinoma cell line has been published previously (22), but the mechanism whereby MMP-1 binds to these cells has not been described. In the present study, we show that MMP-1 binds to EMMPRIN, a tumor cell surface glycoprotein previously shown to induce synthesis of MMP-1 and other MMPs by fibroblasts (9–11) and endothelial cells.[4] We have also shown that an EMMPRIN-MMP-1 complex can be isolated from LX-1 human lung carcinoma cell membranes and that MMP-1 is present on the LX-1 cell surface. A preliminary report has been published suggesting that EMMPRIN becomes localized to invadopodia in human breast carcinoma cells (23). Tumor cell surface EMMPRIN may then be responsible for targeting MMP-1 to invadopodia, thus adding MMP-1 to the impressive list of proteases associated with these invasive structures (6, 17). Although other proteases have been shown to be important in tumor growth and invasion under a variety of conditions, it is likely that MMP-1 is crucial for penetration of fibrous tissues because of its ability to degrade fibrillar collagen as shown, for example, in endothelial cell invasion (24) and tumor cell invasion (25) of collagen gels. Thus localization of MMP-1 on the tumor cell surface via interaction with EMMPRIN would facilitate these invasive processes.

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[4] Unpublished observations.

[CANCER RESEARCH 60, 892–895, February 15, 2000]

Advances in Brief

Gene Promoter Hypermethylation in Tumors and Serum of Head and Neck Cancer Patients[1]

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Abstract

Promoter hypermethylation is an important pathway for repression of gene transcription in cancer cells. We analyzed aberrant DNA methylation at four genes in primary tumors from 95 head and neck cancer patients and then used the presence of this methylation as a marker for cancer cell detection in serum DNA. These four genes were tested by methylation-specific PCR and included: *p16 (CDKN2A)*, O^6-methylguanine-DNA-methyltransferase, glutathione S-transferase P1, and death-associated protein kinase (DAP-kinase). Fifty-five % (52 of 95) of the primary tumors displayed promoter hypermethylation in at least one of the genes studied: 27% (26/95) at *p16*, 33% (31 of 95) at O^6-methylguanine-DNA-methyltransferase; and 18% (17 of 92) at *DAP-kinase*. No promoter hypermethylation was observed at the *glutathione S-transferase P1* gene promoter. We detected a statistically significant correlation between the presence of *DAP-kinase* gene promoter hypermethylation and lymph node involvement ($P = 0.014$) and advanced disease stage ($P = 0.016$). In 50 patients with paired serum available for epigenetic analysis, the same methylation pattern was detected in the corresponding serum DNA of 21 (42%) cases. Among the patients with methylated serum DNA, 5 developed distant metastasis compared with the occurrence of metastasis in only 1 patient negative for serum promoter hypermethylation ($P = 0.056$). Promoter hypermethylation of key genes in critical pathways is common in head and neck cancer and represents a promising serum marker for monitoring affected patients.

Introduction

HNSCC[4] cancer remains a morbid and often fatal disease. The overall survival has not changed in recent years, despite extensive research on the biological and molecular features of HNSCC. Among the more pressing problems in clinical management are the lack of early detection and the high incidence of local-regional recurrence, even with aggressive surgical therapy (1). Therefore, it is important to develop new molecular targets to be used as diagnostic and prognostic indicators.

An important mechanism for gene transcriptional inactivation is hypermethylation at the CpG islands within the promoter regions (2). Some tumor suppressor genes such as *p16, VHL,* and *MLH1* have been found to harbor promoter hypermethylation associated with loss of protein expression in cancer cells (3–5). Several tumor types have also shown aberrant methylation at CpG islands in other genes, including the detoxifying gene *GSTP1* (6), the DNA repair gene *MGMT* (7), and the potential metastasis inhibitor gene *DAP-kinase* first occurrence. (8). The presence of epigenetic methylation might also be useful as a molecular target for tumor cell detection.

The presence of abnormally high DNA concentrations in the serum of patients with neoplasms of various types was described decades ago (9). Recent publications have demonstrated the tumor origin of this DNA in cancer patients by confirming the presence of tumor-specific molecular abnormalities in the serum. K-*ras* or *p53* mutations have been detected in the serum of colorectal (10, 11), pancreas (12), and breast (13) cancer cases and N-*ras* mutations in some hematological diseases (14). Other DNA abnormalities, such as loss of heterozygosity) and MI, have also been reported in the serum of head and neck (15), lung (16, 17), renal (18), and breast (13) cancer patients. Moreover, recent studies have demonstrated the presence of gene promoter hypermethylation in the serum DNA of lung (8), liver (19), and breast (13) cancer patients. However, the clinical significance of these observations is still not understood.

In the present study, we have analyzed the promoter hypermethylation pattern of the *p16, MGMT, GSTP1,* and *DAP-kinase* genes in the tumor DNA of 95 head and neck primary tumors. The methylation patterns found in the tumors were used as molecular markers for cancer cell detection in the paired serum DNA. Almost half of the HNSCC patients with methylated tumors were found to display these epigenetic changes in the paired serum.

Materials and Methods

Sample Collection and DNA Extraction. Ninety-five primary tumors where collected from patients diagnosed with HNSCC between the years 1993 and 1999. Patients were treated at the Johns Hopkins University School of Medicine or at the Wayne State University. Fresh tumors were obtained from surgical resection of the HNSCC patients. Serum samples were collected from the same patients at diagnosis and stored at −80°C. Tumor and serum DNA was prepared as described previously (15). A single follow-up serum was collected at 6 to 72 months after treatment in nine patients from Johns Hopkins University School of Medicine.

Bisulfite Treatment. DNA from tumor and serum specimens was subjected to bisulfite treatment, as described previously (20). Briefly, 1 μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using the Wizard purification resin (Promega Corp.), again treated with NaOH, precipitated with ethanol, and resuspended in water.

MSP. The modified DNA was used as a template for MSP. DNA methylation patterns in gene CpG islands were determined by chemical modification of unmethylated cytosines to uracil and subsequent PCR using primers specific for either methylated or the modified unmethylated sequences (20). Appropriate negative and positive controls were included in each PCR reaction. Primer sequences for the *p16* (20), *MGMT* (7), *DAP-kinase* (8), and

Received 10/5/99; accepted 1/4/00.

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[1] Supported by ROI DE012588-01. M. S-C. and M. E. are recipients of a Spanish Ministerio de Educacion y Cultura Award. J. G. H. and J. J. are Valvano Foundation Scholars.

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[4] The abbreviations used are: HNSCC, head and neck squamous cell carcinoma; MGMT, O^6-methylguanine-DNA-methyltransferase; GST, glutathione *S*-transferase; DAP-kinase, death-associated protein kinase; MI, microsatellite instability; MSP, methylation-specific PCR.

Expression of the extracellular matrix metalloproteinase inducer (EMMPRIN) and the matrix metalloproteinase-2 in bronchopulmonary and breast lesions

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Running title: EMMPRIN in lung and breast tumors.

Key words: Metalloproteinases, tumor invasion, breast cancer, lung cancer.

Summary

Tumor cells interact with stromal cells via soluble or cell-bound factors stimulating the production of matrix metalloproteinases (MMP), a group of enzymes largely involved in the extracellular matrix remodeling in tumor invasion. Among these factors, Extracellular Matrix MetalloProteinase Inducer (EMMPRIN) has been shown to stimulate *in vitro* the fibroblast production of various MMPs such as interstitial collagenase (MMP-1), stromelysin (MMP-3) and gelatinase A (MMP-2). In this study, the EMMPRIN protein was detected by immunohistochemistry prominently in malignant proliferations of the breast and the lung. It was present at the surface of both tumoral epithelial and peritumoral stromal cells. Since previous studies have demonstrated that stromal cells do not express TCSF mRNAs, it is very likely that EMMPRIN is bound to stromal cells via a specific receptor. Moreover, our observations demonstrated that the peritumoral stromal cells strongly express MMP-2. Taken together, our results show that EMMPRIN is an important factor in tumor progression by causing tumor-associated stromal cells to increase their MMP-2 production, thus facilitating tumor invasion and neoangiogenesis.

Introduction

Tumor invasion and metastasis are the result of a multistep process including basement membrane disruption, stromal infiltration, intravasation and extravasation, and invasion of a target organ by tumor cells. All these processes require the degradation or remodeling of basement membranes components and of extracellular matrix macromolecules by proteolytic enzymes. Among these proteinases, matrix metalloproteinases (MMPs) are particularly implicated in the metastastic cascade because of their broad spectrum of substrates (Chambers and Matrisian, 1997). One member of the MMP family, MMP-2 is known to play a crutial role in tumor invasion because of its ability to degrade basement membrane collagen. Many studies have demonstrated that increased MMP-2 expression is correlated with the invasive properties of tumor cells *in vitro* (Ura *et al.*, 1989; Gilles *et al.*, 1994) and with the malignant phenotype *in vivo* (Garbisa *et al.*, 1990; Nawrocki *et al.*, 1997). *In vivo* observations have showed that in most carcinomas, stromal cells are the principal source of production of MMP-2 (Poulson *et al.*, 1992; Polette *et al.*, 1993; Poulson *et al.*, 1993; Pyke *et al.*, 1993) . Even though quiescent fibroblasts generally produce relatively low amounts of MMPs (Polette and Birembaut, 1996), it is likely that tumor-associated fibroblasts are stimulated to produce the elevated levels of MMPs usually present in malignant tumors.

Tumor cells may interact with stromal cells via soluble or cell-bound factors, stimulating MMP production. The best characterized of these factors, a tumor collagenase stimulatory factor (TCSF), recently renamed Extracellular Matrix MetalloProteinase Inducer (EMMPRIN) was originally isolated from LX-1 human pulmonary carcinoma cell line (Ellis *et al.*, 1991; Nabeshima *et al.*, 1991) EMMPRIN is a transmembrane glycoprotein of 58 kDa and has been identified as a member of the immunoglobulin superfamily (Biswas *et al.*, 1995). EMMPRIN has been shown to stimulate fibroblast production of interstitial collagenase (MMP-1), stromelysin (MMP-3) and gelatinase A (MMP-2) (Kataoka *et al.*, 1993; Guo *et al.*, 1997). EMMPRIN is observed to be present in normal tissues such as epidermis, retinal pigment epithelium, breast lobules and ductules suggesting that EMMPRIN may have a physiological role in tissue remodeling by causing induction of stromal MMPs (De Castro *et al.*, 1996; Marmorstein *et al.*, 1996; Finneman *et al.*, 1997). However, EMMPRIN expression is more

prominently found in tumor proliferations. Indeed, EMMPRIN protein has been detected by immunohistochemistry in malignant tumor cells of skin, bladder and breast carcinomas (Muraoka *et al.*, 1993; Zucker and Biswas, 1994; Van den Oord *et al.*, 1997). Futhermore, EMMPRIN transcripts are expressed by tumor cells of lung and breast carcinomas whereas expression of these mRNAs are undetectable or very weak in normal tissues and benign lesions (Polette *et al.*, 1997). Taken together, all these data suggest that EMMPRIN participates in tumor progression by stimulating the synthesis of specific MMPs by peritumoral fibroblasts. Since previous immunohistochemical studies employing a monoclonal antibody directed against EMMPRIN (E11F4) have shown that this factor is localized to the outer surface of several cancer cell lines Ellis *et al.*, 1989; Van den Oord *et al.*, 1997), it seems likely that tumor cell-stromal cell contact is necessary for EMMPRIN-mediated regulation of MMPs. However, the mecanism of action of EMMPRIN *in vivo* is still unclear.

This prompted us to examine by immunohistochemistry the distribution of EMMPRIN and MMP-2 in regard to the persistance of intact basement membrane which could be an obstacle for the MMP induction by cell-cell contact in various normal, benign and malignant proliferations of the breast and the lung.

Material and methods

Source of tissue

Tissues were obtained from 4 normal bronchi, from 20 lungs resected for squamous cell carcinoma (14 cases) and adenocarcinoma (6 cases), from 8 fibroadenomas of the breast, from 4 intraductal lesions of the breast, and from 20 ductal invasive breast carcinomas (4 of grade 1, 12 of grade 2 and 4 of grade 3, according to the Scarf and Bloom classification) .

Immunohistochemistry

Fresh samples were frozen in liquid nitrogen, cut at -20°C in a Reichert-Jung 2800 Frigocut cryostat (Germany) at a thickness of 5-8 µm and transferred onto gelatin-coated slides. They were washed in phosphate-buffered-saline (PBS) (2 washes, 5 min each) and incubated

with specific antibodies using an indirect immunofluorescence technique. In a first step, non specific binding was blocked with 3 per cent bovine serum albumin (BSA; w/v) in PBS for 30 min. Serial sections were then incubated overnight at 4°C in a moist chamber with the monoclonal antibodies against EMMPRIN (G6.2 at a concentration of 10 µg/ml, Chemicon, Temecula), and MMP-2 (CA 406 at a concentration of 2.5 µg/ml, Oncologix, Gaitherburg) . After two washes in PBS for 5 min and one wash in PBS-BSA for 5 min, the sections were incubated with anti-mouse IgG biotinylated complex (Amersham International, U.K.) at a 1: 50 dilution in PBS-BSA solution for 60 min. Then sections were treated by streptavidin-fluorescein isothiocyanate (FITC) (Amersham International, U.K.) at a 1: 50 dilution in PBS for 30 min.

A double immunostaining was performed for the simultaneous localization of EMMPRIN and type IV collagen. For this double immunostaining, two sucessive labeling reactions for EMMPRIN and type IV collagen were chained up as follows: (i) detection of EMMPRIN using the monoclonal antibody G6.2 at a concentration of 10 µg/ml in PBS-BSA solution; (ii) F(ab')2-fragments anti-mouse IgG-digoxigenin (Boehringer Mannheim Biochemical, Germany) at a concentration of 4µg/ml in PBS solution; (iii) anti-digoxigenin fluorescein Fab fragments (Boehringer Mannheim Biochemical, Germany) at a concentration of 1.3 µg/ml in PBS-BSA solution; (iv) detection of type IV collagen using a rabbit biotinylated polyclonal antibody (Institut Pasteur, France) diluted 1:1000 in PBS-BSA solution; (v) streptavidin-Texas Red conjugate (Amesham, U.K.) at a 1:50 dilution in PBS.

We tested the absence of cross-reactivity either by omitting the incubation step with the primary antibody, or by replacing the primary antibody with a non-immune IgG. The sections were counterstained with Harris hematoxylin solution for 10 s., mounted in Citifluor antifading solution (Agar, U.K.) and observed with an Axiophot microscope (Zeiss, Germany) using epifluorescence for conventional microscopy or under a confocal laser scanning microscope (Biorad MRC 600).

Results

EMMPRIN detection (Table1)

EMMPRIN was detected in all normal and tumoral tissues of the breast and lung (table 1). In the normal ducts and lobules and in fibroadenomas of the breast, EMMPRIN was frequently confined at the apical membrane of epithelial cells (figures 1a, b). In the same way, in the normal bronchi, there was an accumulation of EMMPRIN at the apical pole of the ciliated cells, as confirmed by confocal microscopy examination (figures 1c, d). In these normal or benign conditions, EMMPRIN was also detected by weak labeling at the basal pole of epithelial cells. In tumoral clusters, EMMPRIN was highly expressed in all malignant cells (figures 2a, b) with more intense staining on the cells located at the periphery of the well-differenciated nests (figure 2c). Positive staining was distributed at the outer cell membrane of these cancer cells with a punctiform pattern (figure 2d). Furthermore, EMMPRIN was also found in some stromal cells in breast and lung carcinomas close to tumor cells (figure 2a). There were positivities at the cell surface of isolated elongated cells considered as fibroblasts. Moreover, some endothelial cells displayed spotty labelings on their cell membrane (figure 2e). Since our previous data have shown no EMMPRIN mRNAs in peritumoral stromal cells (Polette *et al.*, 1997), these observations support the hypothesis that stromal cells would bind the EMMPRIN produced by epithelial tumor cells.

Colocalization of EMMPRIN and type IV collagen

We next investigated whether basement membrane integrity could be an obstacle to the diffusion of EMMPRIN from tumor cells. Using double labeling of immunofluorescence, we found that the stromal positivities of EMMPRIN were not necessarily associated with the absence of type IV collagen around tumor clusters, suggesting that basement membrane integrity is not a limiting factor to EMMPRIN translocation (figure 2f).

Colocalization of EMMPRIN and MMP-2

Since EMMPRIN has been shown to stimulate fibroblast production of several MMPs, we looked at MMP-2 localization in relation with that of EMMPRIN. As expected, MMP-2

protein was detected in the benign neoplasms and malignant lesions whereas this enzyme was rarely detected in normal tissues adjacent to tumors. In the carcinomas, this MMP-2 was found both in cancer and peritumoral stromal cells whereas in fibroadenomas it was only present in some sparse fibroblasts at distance from the proliferating ducts (Table 1). In all carcinomas, we observed that the presence of EMMPRIN in/on stromal cells coincided with the detection of MMP-2 in the same cells (figures 2g, h).

Discussion

This immunohistochemical study demonstrates epithelial expression of EMMPRIN in normal tissues and in various benign and malignant proliferations of the breast and the lung. These data are in agreement with previous reports using a different antibody (E11F4) in the mammary gland [19]. With the E11F4 antibody, De Castro *et al* (1996) have found that normal keratinocytes express EMMPRIN *in vitro* and *in vivo* . In contrast, Muraoka *et al* (1993) have detected the presence of EMMPRIN mostly in urothelial malignant cells and considered that this cell surface protein could be a tumor marker for bladder cancers. There is also an apparent discrepancy between our immunohistochemical data and our first observations with *in situ* hybridization on the same kind of tumors. Indeed, EMMPRIN mRNA transcripts have been detected only in malignant tumor cells but never in the normal tissues and benign proliferations (Muraoka *et al.*, 1993). Nevertheless, in these latter samples, Northern blot analysis has revealed some weak positivities which could reflect a low transcriptional activity. Thus, the immunohistochemical detection of the protein EMMPRIN in normal epithelial cells may correspond to a low basal level of expression and/or a short half-life of EMMPRIN mRNAs in normal tissues and benign lesions undetectable with *in situ* hybridization. An aspect of the biochemical properties of EMMPRIN that we have demonstrated and needs emphasis is that the EMMPRIN molecule must be glycosylated to function as an inducer of MMP synthesis. It is possible the EMMPRIN in normal , in contrast to tumor tissues, is not glycosylated and, hence, lacks functional activity as an MMP inducer (Biswas *et al.*, 1995; Guo *et al.*, 1997). Even though the EMMPRIN expression in normal epithelial cells was weak, it might be

representative of a physiological role in tissue remodeling under particular conditions. In contrast, extensive expression of EMMPRIN in invasive tumor cells of breast and lung carcinomas clearly support its role in tumor invasion. Moreover, the strong immunoreactivity for EMMPRIN in well differentiated tumor clusters versus poorly differentiated nests emphasizes that EMMPRIN is implicated in the early stages of tumor progression.

Besides this distribution of EMMPRIN on cancer cells, we also found this protein in peritumoral stromal cells in malignant lesions. Since the biosynthesis of EMMPRIN by fibroblasts has never been reported in previous *in vitro* and *in vivo* studies (Ellis *et al.*, 1989; Muraoka *et al.*, 1993; Zucker and Biswas, 1994; Van den Oord *et al.*, 1997), it is very likely that these peritumoral stimulated cells are able to bind the EMMPRIN secreted by epithelial cancer cells. The absence of EMMPRIN protein both in fibroblasts and endothelial cells of normal mammary and lung tissues suggest that peritumoral stromal cells may have acquired the ability to express specific receptors for EMMPRIN during tumor progression. The binding of EMMPRIN to stromal cells could then induce their MMP production. Indeed, we and others have previously showed that stromal cells are the principal source of several MMPs such as MMP-2. In this study, we observed that the presence of EMMPRIN is associated with the expression of MMP-2 in all the cases of carcinomas. Indeed, in our comparative study using serial sections, we detected a concomitant expression of EMMPRIN and MMP-2 in tumoral and stromal cells. Furthermore, we have clearly observed a colocalization of EMMPRIN protein and MMP-2 in tumoral-associated fibroblasts. These observations are consistent with the hypothesis that EMMPRIN expressed by cancer cells stimulates stromal MMP producton via specific receptors.

Even though the cellular mechanism of action of EMMPRIN is not yet well understood, several hypothesis can be drawn from our results. In general, it seems that the plasma membrane localization of EMMPRIN at the periphery of tumor clusters serves to restrict its bioactivity to cells in close proximity. Indeed, EMMPRIN attached to the plasma membrane via a transmembrane domain could interact with a cell surface receptor present on peritumoral stromal cells via an extracellular domain (Nabeshima *et al.*, 1991; Biswas *et al.*, 1995). In this instance, the presence and/or the persistence of an intact basement membrane would represent

an obstacle for the direct stimulating effect of EMMPRIN on fibroblasts adjacent to epithelial cells. This may partly explain the absence of concomitant stromal expression of MMP-2 in normal tissues and their limited expression in benign neoplasms. This could also reflect the apparent inability of stromal cells in normal tissues to express specific receptors and consequently to respond to EMMPRIN by an increased MMP-2 production. Independent of the presence of an intact basement membrane around tumor clusters, some invasive tumor cells seemed to acquiere the capacity to shed EMMPRIN as components of membrane vesicles, thereby enabling EMMPRIN activity to extend beyond the direct cell contact effect. We, indeed, found in our malignant tumors the presence of EMMPRIN in the connective tissue close to tumor cells, even in the presence of type IV collagen deposited between tumor clusters and the stroma. This shed form of EMMPRIN could therefore bind to specific receptors present only in peritumoral stromal cells and stimulate them to produce MMPs in carcinomas.

In conclusion, our results describing an intense expression of EMMPRIN in cancer tissue clearly implicate EMMPRIN in tumor invasion. The detection of EMMPRIN protein on peritumoral stromal cells and in fine vesicules in the connective tissue also support the hypothesis that this factor could be shed from tumor cells and fixed by tumor-associated stromal cells. This would further stimulate the stromal production of MMP-2 facilitating tumor invasion and neoangiogenesis. Taken together these results advance our understanding of the cooperation between cancer and host cells during the invasion and metastatic process.

Acknowledgements to the LIONS Club of Soissons for supporting this work. Chemicon (Temecula, CA) for generously providing the antibody to EMMPRIN.

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Legends of figures

Table 1: Distribution of EMMPRIN and MMP-2 in bronchopulmonary and breast lesions.

Reactivity staining grader: +++, extensive; ++, moderate; +, faint; -, negative.

grades I, II, III : Scarf and Bloom classification.

Figure 1: EMMPRIN localization in normal and benign proliferations of breast and lung carcinomas. EMMPRIN is present on the outer membrane of epithelial cell with a dense labeling at the apical cell membrane (arrows) in normal lobule breast (a) (Bar=70µm). In fibroadenoma of the breast, EMMPRIN is also prominently found at the apical pole of the epithelial cells by confocal microscopy analysis (arrows) (b) (Bar=20µm). By confocal microscopy analysis, EMMPRIN is located in tha apical compartment of the epithelial cells in the normal airways (arrows) (c) (Bar=40µm) or even concentrated at the apical surface of the epithelial cells (arrows) (d) (Bar=10µm).

Figure 2 : EMMPRIN detection in breast and lung carcinomas. EMMPRIN is localized in cancer cells (T) both in breast (a) and in lung (b) carcinomas (Bars=70µm). By confocal microscopy analysis, the EMMPRIN labeling is preferentially distributed at the periphery of well-differentiated clusters (T) (c) and is detected around cancer cells (d) (Bars=40µm, 4µm). EMMPRIN is also found both in fibroblasts (arrows) (a) and endothelial cells (arrow) (e) close to tumor nests (T) (Bar = 70µm). The stromal positivities of EMMPRIN (arrow) is detected near tumor clusters where type IV collagen in red is present (f) (Bar=70 µm). In the same area, EMMPRIN (g) and MMP-2 (h) are detected in both tumor (T) and stromal cells (arrows) (Bars=70 µm).

Histological type and differentiation stade (number of cases)	EMMPRIN		MMP-2
	Epithelium	Stroma	Stroma
Normal bronchi (4)	+	-	-
Adenofibromas of the breast (8)	+	-	-
Intraductal lesions of the breast (4)	++	+	+
Squamous cell carcinomas of the lung			
Well differentiated (3)	+++	+	+
Moderately differentiated (4)	+++	++	++
Poorly differentiated (7)	+	+++	+++
Adenocarcinomas of the lung			
Well differentiated (3)	++	+	+
Poorly differentiated (3)	+	++	+++
Adenocarcinomas of the breast			
Well differentiated (grade I) (4)	++	+	+
Moderately differentiated (grade II) (12)	++	+	++
Poorly differentiated (grade III) (4)	+	++	+++

Tumorigenic Potential of Extracellular Matrix Metalloproteinase Inducer (EMMPRIN)[1]

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[1]Supported by a Department of Defense Breast Cancer Grant (DAMD 17-95-5017) and a Department of Veterans Affairs Merit Review Grant

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The abbreviations used are: ECM, extracellular matrix; EMMPRIN, extracellular matrix metalloproteinase inducer; GFP, green fluorescent protein; MMP, matrix metalloproteinases; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; ELISA, enzyme linked immunosorbent assay.

ABSTRACT

EMMPRIN, a glycoprotein present on the cancer cell plasma membrane, enhances fibroblast synthesis of matrix metalloproteinases (MMPs). In this report we have demonstrated a role for EMMPRIN in cancer progression. Human MDA-MB-436 breast cancer cells, which are tumorigenic but slow growing in vivo, were transfected with EMMPRIN cDNA and injected orthotopically into mammary tissue of female nu/nu mice. Green fluorescent protein (GFP) was utilized to visualize metastases. Breast cancer cell clones transfected with EMMPRIN/GFP cDNA were considerably more tumorigenic and invasive than GFP- or plasmid- transfected cancer cells. Increased MMP expression was demonstrated in EMMPRIN enhanced tumors.

INTRODUCTION

Extracellular Matrix MetalloPRoteinase INducer (EMMPRIN) was originally designated Tumor Collagenase Stimulating Factor (TCSF) by Biswas et al. following isolation and purification of the 58 kDa glycoprotein from the plasma membrane of cancer cells and demonstration of its function in stimulating fibroblast synthesis of interstitial collagenase (MMP-1) (1). The subsequent finding that EMMPRIN also induced fibroblast synthesis of gelatinase A (MMP-2) and stromelysin-1 (MMP-3) indicated a more general effect on production of MMPs (2). Recent studies have documented the capacity of recombinant EMMPRIN or EMMPRIN purified from cancer cells to stimulate fibroblast production/secretion of stromelysin-1, interstitial collagenase, and gelatinase A in vitro (2), (3). The demonstration by in situ hybridization (mRNA localization) that peritumoral fibroblasts synthesize most of the MMPs (collagenases, gelatinases, stromelysins, MT-MMPs) in human tumors rather than the cancer cells themselves has ignited interest in the role of EMMPRIN in tumor dissemination (4), (5). The association of intense EMMPRIN expression in neoplastic cells within invasive human tumors (6) further supports a role for EMMPRIN in cancer dissemination. These data are consistent with a central function for EMMPRIN in stimulating stromal cell production of MMPs which, following pericellular activation, directly degrade extracellular matrix (ECM) (1).

In the current study we have examined the function of EMMPRIN in a cancer model in immunodeficient mice. Human MDA-MB-436 breast cancer cells which are tumorigenic, estrogen independent, moderately invasive in vitro, but slow growing in vivo (7), were transfected with EMMPRIN cDNA and injected orthotopically into the mammary fat pad of nude mice. We took advantage of the observation that the 29 kDa green fluorescent protein (GFP) of the jelly fish Aequoria victora retains its fluorescent properties when recombinantly expressed in eukaryotic cells (8) along with EMMPRIN cDNA and can be used as a powerful marker for gene expression and cancer dissemination in vivo. Cancer cells transfected with both EMMPRIN cDNA and GFP cDNA were compared to cancer cells transfected with GFP cDNA alone for tumorigenic behavior.

MATERIALS AND METHODS

Reagents

Restriction enzymes were purchased from Stratagene (La Jolla, CA). EMMPRIN was purified from LX-1 lung cancer cells using affinity column chromatography (9). Monoclonal antibodies to EMMPRIN (clone 1G6.2) were produced in collaboration with Dr. Dembro at Chemicon, International, Inc. (Temecula, CA).

Cell lines and Culture Conditions

Human MDA-MB-436 breast cancer cells were maintained in Richter's Improved Minimal Essential Medium supplemented with 10% donor calf serum (7). Immunostaining of MDA-MB-436 cells was performed using a primary mouse monoclonal antibody to EMMPRIN (1G6.2) and a secondary goat anti-mouse IgG (H&L) horseradish peroxidase labeled antibody.

Construction of Plasmids and Transfection into Cells

A 1.6 kb cDNA (1), representing the entire EMMPRIN sequence encoding 269 amino acid residues, was placed at an EcoR I site under the control of the CMV promoter in pcDNA3 (Invitrogen, Carlsbad, CA). To facilitate identification of transfected cells in vitro and metastases in vivo, green fluorescent protein (GFPmut1 variant) cDNA (Clontec Lab, Inc., Palo Alto, CA) was inserted into the EMMPRIN-containing plasmid. The GFP cDNA along with a separate upstream CMV promoter from pEGFP-C1 plasmid (Clontec Lab, Palo Alto, CA) was inserted into the EMMPRIN expression vector between Not I and Xho I sites as shown in Figure 1A. An additional polyadenylation (PA) signal from pSG5 (Stratagene) was placed downstream of the EMMPRIN gene to provide balanced expression of both recombinant genes under control of CMV promoters. The resulting plasmid was named EMMPRIN/GFP. As a control plasmid, GFP cDNA alone was subcloned into pcDNA3 without EMMPRIN cDNA. In experiment 2, EMMPRIN cDNA was subcloned into pcDNA3 without GFP; the control plasmid was pcDNA3 alone.

The human MDA-MB-436 breast cancer cell line was stably transfected using the calcium phosphate precipitation method (10). Selected G418-resistant clones were screened by fluorescent appearance (Figure 2A) using a Nikon microscope equipped with a Xenon lamp power supply and a GFP filter set. Fluorescent positive clones were further analyzed by Northern blot analysis probed with an EMMPRIN cDNA fragment.

RNA Isolation and Northern Blot Hybridization

Total RNA was extracted from MDA-MB-436 cells stably transfected with desired plasmids by guanidine solubilization, phenol/chloroform extraction, and serial precipitation (1), (11). Approximately 20 μg of total RNA was resolved by denaturing gel electrophoresis followed by Northern transfer to nylon membranes (Schleicher and Schuell, Keene, NH). Blots were hybridized to ^{32}P-radiolabeled EMMPRIN cDNA (1.7 Kb) at 42°C as described (11) and analyzed after overnight exposure with an intensity screen at -80°C. The amount of the samples applied to the lanes was normalized by β-actin RNA.

Labeling of RNA Probes

Antisense and sense digoxigenin (DIG)-labeled RNA probes for human EMMPRIN and mouse gelatinase A, and gelatinase B were synthesized by reverse transcribing 1μg of cDNA from a PCR reaction that had used gene-specific primers that contain the T7 or T3 phage promoter sequence followed by 20-25 bases of the mRNA sequence (12). In vitro transcription of the amplified DNA template was performed using the digoxigenin RNA labeling kit (Boehringer-Mannheim). Labeled probes were purified and sequences were verified.

In situ hybridization

Serial sections of paraffin-embedded mouse tumors were prepared for in situ hybridization according to the method of Komminoth (13). Slides were processed for immunodetection employing anti-DIG alkaline phosphatase antibody and then incubated with substrate solution (Boehringer-Mannheim Wash and Block Set).

Cell Proliferation In Vitro

Cell proliferation assays were performed by plating MDA-MB-436 cells at 4×10^4 cells per well (Costar, Corning, NY) and then switched to serum-free media. After 48 hours, serum-enriched media was added back and cells were cultivated for 4 additional days. Cell counts were performed daily.

Tumor Formation in Mice and Preparation of Tissue Extracts

Four week old female athymic NCr nu/nu mice were obtained from Taconic Farms (Germantown, NY). Cancer cells (1×10^6) were injected into the mammary fat pad of nude mice. Tumor growth was monitored weekly. Tumor volume was calculated using the formula: $(\text{length})(\text{width})^2/2$. Tissues from autopsies were prepared for histology and stored in liquid nitrogen. The extraction procedure for tumor tissue, involved detergent and heat-extraction steps (14).

Immunoassays, Immunoblotting, Gelatin Substrate Zymography, and Protein Studies

Primary cell cultures were transferred to serum-free media for 2 days; spent media was collected and tested by gelatin zymography. Gelatinase A, stromelysin-1, and gelatinase B were measured using sandwich ELISA formats as we have described in detail previously (15). Gelatin substrate zymography was preformed in 10% polyacrylamide gels that had been cast in the presence of 0.1% gelatin (NOVEX, San Diego, CA) (15), (16). Protein determinations were made using the bicinchoninic acid reagent (Pierce, Rockford, IL).

Analysis of variance and Student's t-test were employed to compare differences between groups in various experiments; $p<0.05$ was considered significant. Survival experiences between groups were compared by the Wilcox chi-square test.

RESULTS

Cell Transfection and Proliferation

Northern blot analysis using EMMPRIN cDNA as a probe detected 20 fold enhanced EMMPRIN expression by EMMPRIN-transfected cells as compared to GFP or non-transfected cells (Figure 1B). Immunostaining of MDA-MB-436 cells using specific mouse monoclonal antibodies to EMMPRIN documented intense staining of EMMPRIN/GFP transfected cells and infrequent weak staining of GFP-transfected or vector transfected cells (data not shown).

There were no significant differences in cell doubling times between GFP and EMMPRIN/GFP cDNA transfected cells (~18 hours) in media with or without serum. This data is inconsistent with EMMPRIN acting as an autocrine growth factor for tumor cells in vitro.

Tumor Growth in Nude Mice

Three independent experiments, each employing a different clone of EMMPRIN-transfected MDA-MB-436 cells, were performed. In experiments 1 and 2, the GFP-alone or vector transfected clones did not form palpable tumors by the time of the experiment's termination at 12 weeks; however, ~.01 cm^3 non-invasive tumors were identified at autopsy in 18/18 mice. In contrast, the EMMPRIN/GFP transfected clones formed palpable breast tumors at the site of mammary injection by week 6 in 18/18 mice which grew progressively to >1.7 cm^3 in diameter by week 12 at which time the animals were sacrificed. Histologic examination revealed local cancer invasion, but no metastases. EMMPRIN/GFP and GFP expressing tumors expressed green fluorescence when examined grossly with a fluorescent light.

Experiment 3: Groups of 10 mice were injected with transfected MDA-MB-436 cells into mammary tissue. The tumors emanating from the EMMPRIN/GFP cDNA transfected MDA-MB-436 cells grew relatively rapidly and all mice expired or had to be sacrificed within 12 weeks (Figure 2B and 3A); extensive metastases to the liver, lung, pleura, spleen, lymph nodes, and mesentery were present in 3/10 mice. In contrast, injection of the GFP cDNA transfected tumor cells into mice resulted in tumors that grew much more. Tumor diameter was <0.3 cm^3

and no metastases were noted at week 15. One mouse in the GFP-transfected group developed a 1.4 cm^3 primary tumor by week 12.

Gelatinolytic Activity Extracted from Tumor Tissues and Cells

Gelatin zymograms of conditioned media from cultivated MDA-MB-436 cells (Figure 3B- left panel) revealed that cells transfected with GFP or EMMPRIN/GFP cDNA secreted equivalent amounts of 72 kDa gelatinase A; gelatinase B was not detected. Tumor extracts from EMMPRIN/GFP injected mice displayed intense gelatinolytic bands localized at 105 kDa, 92 kDa, 72 kDa, and 64-62 kDa (Figure 3B- right panel). The 105 kDa band is consistent with mouse latent gelatinase B; human latent gelatinase B migrates at 92 kDa (17). The 72 kDa and 62 kDa gelatinolytic bands could represent human or mouse latent and activated gelatinase A, respectively. Tumor extracts from GFP alone-injected mice revealed weaker gelatinolytic bands than EMMPRIN/GFP injected mice.

Histochemistry/In situ Hybridization

Hematoxylin and eosin staining of resected breast masses revealed extensive replacement of normal mammary tissue with carcinoma in tumors originating from mice injected with EMMPRIN/GFP or GFP transfected MDA-MB-436 cells. In situ hybridization of tumor tissue from mice injected with EMMPRIN/GFP-transfected cells revealed widely distributed, specific staining with EMMPRIN in cancer cells; surrounding benign appearing mammary ducts also expressed EMMPRIN (Figure 4). Gelatinase A mRNA was found in both cancer cells and the surrounding non malignant tissue (muscle, fat, and benign-appearing mammary ducts). There was specific staining with the gelatinase B antisense riboprobe in cancer cells, but not as widely distributed as gelatinase A; intense periductal staining was noted in surrounding normal tissue. Similar results were found on examination of metastatic tumors in the EMMPRIN/GFP treated mice. Specific staining was abolished by pretreatment of tissues with RNAase (data not shown). In the GFP alone-transfected tumors, virtually no EMMPRIN or gelatinase A staining was seen.

Focal staining for gelatinase B was noted in GFP tumor tissue, not in surrounding normal tissue. No staining was detected in any of the tumor tissues that were hybridized with EMMPRIN, gelatinase A, or gelatinase B sense probes (data not shown).

DISCUSSION

The current report describes a direct effect of EMMPRIN expression on tumorigenicity in an animal model. Transfection of EMMPRIN cDNA or EMMPRIN/GFP cDNA into human breast cancer cells resulted in marked enhancement of tumor growth in nude mice after orthotopic injection of tumor cells. High levels of EMMPRIN, gelatinase A, and gelatinase B mRNA expression within tumors was documented by in situ hybridization. Enhanced gelatinase B and gelatinase A were identified in zymograms from extracts of EMMPRIN/GFP-transfected tumors as compared to GFP tumors. It is noteworthy that MDA-MB-436 cells propagated in vitro produce gelatinase A, but not gelatinase B, whereas extracts of tumors in nude mice have higher levels of gelatinase B than gelatinase A. An association between expression of EMMPRIN and gelatinase B in benign and malignant pigment cell skin lesions has been reported (18).

Metastasis following orthotopic injection of tumor cells into nude mice was greater with EMMPRIN/GFP-transfected than with GFP-transfected cells, but the overall rate was low. EMMPRIN expression did not affect tumor cell proliferation in vitro. Based on the established role of EMMPRIN in enhancing MMP synthesis by stromal fibroblasts, it would appear that increased degradation of extracellular matrix permits more rapid tumor growth in vivo. The higher rate of tumor growth with EMMPRIN-transfected cancer cells and the associated matrix degradation may also occur by favored neoplastic cell survival in a tissue stroma environment initially not permissive for tumor growth. Enhanced extracellular matrix degradation may also release growth factor-like fragments of matrix components, resulting in an indirect effect on cell proliferation (19). These studies with EMMPRIN reinforce the notion that cancer dissemination is a multistep process and that protein degradation contributes to the process but is insufficient in itself for tumor metastasis (20).

ACKNOWLEDGMENTS

This article is dedicated to the memory of our friend and colleague, Chitra Biswas, whose career was dedicated to the discovery and exploration of EMMPRIN. Dr. Biswas died in August 1993, but her inspiration continues to guide us in our studies of EMMPRIN.

We thank Dr. Serge Lyubsky for his contribution to the histopathologic studies.

FIGURE LEGENDS

Figure 1A. Schematic illustration of the EMMPRIN/GFP plasmid. A 1.6 kb cDNA representing the entire EMMPRIN sequence was placed at an EcoR 1 site under the control of the CMV promoter in pcDNA 3. GFP cDNA was inserted along with an upstream CMV promoter into the EMMPRIN expression vector between Not I and Xho I sites. A polyadenylation (PA) signal was placed downstream.

Figure 1B. Northern blot analysis of EMMPRIN. ~20 μg of total cellular RNA from plasmid alone-transfected, GFP-transfected, and EMMPRIN/GFP transfected MDA-MB-436 breast cancer cells was size fractionated in a 1% denaturing agarose gel, transferred to a nylon membrane, and incubated with 1.7 kb of ^{32}P-radiolabeled EMMPRIN cDNA as a probe. Blots were analyzed by autoradiography. A single 1.7 kb mRNA transcript corresponding to the known EMMPRIN band was detected at ~20 X greater intensity in EMMPRIN/GFP transfected cells as compared to plasmid alone or GFP transfected cells.

Figure 2A. Identification of green fluorescence in MDA-MB-436 cells. Left panel displays GFP-transfected cells. Middle panel displays EMMPRIN/GFP transfected cells. Right panel displays the expression of EMMPRIN-GFP as a fusion molecule (cDNA controlled by a single CMV promoter) in MDA-MB-436 cells. Fluorescence in the left and middle panels identifies GFP in the cell cytoplasm. GFP fluorescence in right panel identifies the EMMPRIN-GFP fusion protein localized to cell organelles and plasma membranes.

Figure 2B. GFP-transfected tumors are readily visible under fluorescent light. EMMPRIN/GFP transfected MDA-MB-436 breast cancer cells were injected into a NCr nu/nu mouse. Eight weeks later, extensive green colored metastatic tumors (identified with arrows) in the pleura, peritoneum, lymph nodes, liver, and spleen are visible under fluorescent light (left photo). The middle photo demonstrates the same mouse at autopsy illuminated with bright light.

The right photo demonstrates typical tumor size of mice sacrificed at week 15. Tumors from mice injected with EMMPRIN/GFP transfected MDA-MB-436 cells are considerably larger and show prominent blood vessels on the tumor surface as compared to mice injected with GFP-transfected cells. Arrow heads point to tumors.

Figure 3A. MDA-MB-436 breast cancer cells transfected with EMMPRIN/GFP cDNA resulted in enhanced rate of tumor growth after tumor cell implantation into the mammary fat pad of nude mice as compared to GFP-transfected cells. The data represents the mean ± standard error observed in 10 animals in each group. The numbers associated with each symbol refer to the number of mice alive at each time point.

Figure 3B. Comparison of gelatinases produced by MDA-MB-436 cells cultivated in serum-free media and extracts of nude mouse tumors. Spent conditioned media from primary cells cultivated in vitro (left panel) and tumor cell extracts (right panel) were assessed by gelatin substrate zymography. Protein concentration of samples were equalized within each group. Tumor extracts from the EMMPRIN/GFP group had more gelatinolytic activity than GFP-alone (displayed extract from GFP-alone tumor is from the largest tumor in that category).

Figure 4. In situ hybridization of primary tumors from mice injected with EMMPRIN/GFP and GFP-transfected MDA-MB-436 breast cancer cells. Serial sections from tumor tissue (panels 1-4, 9-12) and surrounding non malignant tissue (panels 5-8 and 13-16) were examined. Panels 1 and 9 represent hematoxylin and eosin staining of cancer tissues from EMMPRIN/GFP and GFP tumors, respectively; panels 5 and 13 represent H & E staining of non malignant tissues from EMMPRIN/GFP and GFP tumors, respectively. Cells in the primary tumor mass from mice injected with EMMPRIN/GFP transfected cells revealed widely distributed, specific staining with EMMPRIN, gelatinase A (GLA), and gelatinase B (GLB) antisense riboprobes (panels 2-4). Minimal cell staining was seen in cancer cells from GFP-transfected MDA-MB-436 cells for

EMMPRIN and gelatinase A (panels 10, 11), focal gelatinase B was identified in GFP tumors (panel 12). Non malignant tissues adjacent to the primary tumors from EMMPRIN/GFP mice demonstrated staining for EMMPRIN, gelatinase A, and gelatinase B in mammary ducts (D) and myocytes (M) (panels 6-8). Non malignant tissue from GFP mice revealed no staining for EMMPRIN, gelatinase A, or gelatinase B (panels 14, 15, 16).

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Expression of EMMPRIN (CD147), a cell surface inducer of matrix metalloproteinases, in normal human brain and gliomas: EMMPRIN expressed on glioma cells stimulates production of MMP-2 and MT1-MMP by brain-derived fibroblasts

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Running title: EMMPRIN expression in human gliomas
Key words: EMMPRIN, basigin, MMP, glioma, invasion, angiogenesis

ABSTRACT

EMMPRIN (extracellular matrix metalloproteinase inducer), also called CD147, basigin or M6 in the human, is a member of the immunoglobulin superfamily that is present on the surface of tumor cells and stimulates adjacent fibroblasts to produce matrix metalloproteinases (MMPs). In this study, we investigated expression of EMMPRIN in human normal brain and gliomas, since mouse basigin and chicken HT7, the species homologues of human EMMPRIN, are associated with neuronal interactions and normal blood-brain barrier function, respectively. EMMPRIN expression was detected in all samples of non-neoplastic brain and glioma tissues examined. However, expression levels of EMMPRIN mRNA and protein were markedly higher in gliomas than in non-neoplastic brain. Moreover, mRNA expression levels correlated with tumor progression in gliomas: they were highest in the most malignant form of glioma, glioblastoma multiforme, followed by anaplastic astrocytoma and then low grade astrocytoma in descending order. Also, immunolocalization revealed quite different distributions in non-neoplastic brain and glioma: EMMPRIN was demonstrated only in vascular endothelium in non-neoplastic regions of the brain whereas it was present in tumor cells but not in proliferating blood vessels in malignant gliomas. *In vitro*, coculturing of EMMPRIN-expressing human glioblastoma multiforme cells (U251) with brain-derived human fibroblasts lacking EMMPRIN stimulated production and activation of pro-MMP-2 (gelatinase A). Production of MT1-MMP, an activator of pro-MMP-2, was also stimulated in these cocultures. The stimulation of MMP-2 and MT1-MMP production in cocultures was inhibited by anti-EMMPRIN monoclonal antibody in a dose-dependent manner. Thus, EMMPRIN produced by glioblastoma multiforme cells stimulates expression of pro-MMP-2 and MT1-MMP and, consequently, activation of pro-MMP-2. This activated MMP-2 could be involved in glioma cell invasion or in tumor angiogenesis.

INTRODUCTION

It is known that degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMP) is a crucial step in tissue remodeling and tumor invasion and metastasis (Biswas and Toole, 1987; Liotta et al., 1991). In tumor invasion, not only tumor cells but also host stromal cells, especially fibroblasts, are responsible for MMP production, and the role of tumor cell-fibroblast interactions in regulation of MMP levels in tumors has been demonstrated by several investigators, including ourselves (Biswas, 1982; Dabbous et al., 1983; Goslen et al., 1985; Nabeshima et al., 1994; Himelstein et al., 1994; Ito et al., 1995; Kurogi et al., 1996). Moreover, *in vivo*, *in situ* hybridization studies have clearly demonstrated that most tumor-associated MMPs, such as interstitial collagenase (MMP-1), stromelysin-1 (MMP-3), stromelysin-3 (MMP-11), and gelatinase A (72 kDa gelatinase, MMP-2), are mainly synthesized in peritumoral fibroblasts rather than in tumor cells themselves in breast, colon, lung, skin and head and neck cancers (Basset et al., 1990; Gray et al., 1992; Pyke et al., 1992; Poulsom et al., 1992, 1993; Noel et al., 1994; Majmudar et al., 1994; Okada A et al., 1995; Heppner et al., 1996; Polette et al., 1997). The physiological activator of pro-MMP-2, membrane-type MMP (MT-MMP), is also expressed in the stroma of breast, colon and head and neck cancers (Okada A et al., 1995). Thus, not only production but also extracellular activation of MMPs may be aided by stromal cells in tumors, and indeed recent investigations have shown clearly that stromal MMP production promotes tumor progression (Itoh et al., 1998; Noel et al., 1998; Masson et al., 1998).

Extracellular matrix metalloproteinase inducer (EMMPRIN; previously termed tumor cell-derived collagenase stimulatory factor or TCSF), was discovered by the Biswas laboratory via a functional approach and shown to be a surface molecule on tumor cells that stimulates nearby fibroblasts to produce MMP-1, 2 and 3 (Biswas, 1982; Ellis et al., 1989; Prescott et al., 1989; Nabeshima et al., 1991; Kataoka et al., 1993; Biswas et al., 1995; Guo et al., 1997). The expression levels of EMMPRIN, as determined by immunohistochemistry, are upregulated in urinary bladder, breast and lung carcinomas compared with their normal counterparts, and EMMPRIN has been implicated in progression and invasion in these tumors in these studies (Muraoka et al., 1993; Polette et al., 1997). The cDNA for human EMMPRIN encodes a 269-amino acid residue polypeptide that includes a signal peptide of 21 amino acid residues, a 185-amino acid extracellular domain consisting of two regions characteristic of the immunoglobulin superfamily, followed by a 24-amino acid residue transmembrane domain and a 39-amino acid cytoplasmic domain (Biswas et al., 1995). Recombinant(r)-EMMPRIN isolated from CHO cells transfected with EMMPRIN cDNA is highly glycosylated and has a molecular mass of ~58 kDa, similar to tumor cell surface EMMPRIN. This rEMMPRIN successfully stimulated production of MMP-1, 2 and 3 (Guo et al., 1997). Moreover, the EMMPRIN sequence has been found to be identical to that of human basigin (Miyauchi et al., 1991) and human leukocyte activation-associated M6 antigen (Kasinrerk et al., 1992), the species homologue of rat OX-47 antigen (Fossum et al., 1991), mouse basigin (gp42) (Miyauchi et al., 1991), and chicken blood-brain barrier (BBB) specific HT7 molecule, also known as neurothelin (Seulberger et al., 1990; Schlosshauer and Herzog, 1990), and all of which are now called CD147.

Gliomas are the most common human primary brain tumors, and astrocytic tumors comprise the largest subgroup of these tumors (Kleihues et al., 1993). The most malignant form of gliomas, glioblastoma multiforme (GBM), may occur *de novo* or may result from progression of low grade gliomas: for example astrocytoma may progress to anaplastic astrocytoma, then to GBM. The ability to infiltrate and invade the surrounding normal brain tissue is very characteristic of malignant astrocytic

tumors, such as anaplastic astrocytoma and GBM, and degradation of ECM by MMPs is involved in this invasion (Giese and Westphal, 1996). Among MMPs, MMP-2 expression has been shown to correlate most closely with malignant progression of gliomas *in vivo* and with invasive activity of human glioma cells *in vitro* (Nakano et al., 1995; Sawaya et al., 1996; Uhm et al., 1996; Lampert et al., 1998). However, the role of tumor cell-stromal cell interactions or of EMMPRIN itself in regulation of MMP levels in gliomas has never been explored. Additionally, the species homologues of human EMMPRIN, mouse basigin and chicken HT7, have been shown to be expressed in brain and implicated in neuronal interactions (Fadool and Linser, 1993) and normal blood-brain barrier (BBB) function (Seulberger et al., 1990; Schlosshauer and Herzog, 1990), respectively. Thus, in this study, we investigated EMMPRIN expression in human normal brain and gliomas, and examined its role using *in vitro* cocultures of human glioma cells with brain-derived fibroblasts. The results clearly showed: a) upregulation of EMMPRIN in gliomas, especially in malignant gliomas; b) stimulation by EMMPRIN expressed on glioma cells of fibroblast production of pro-MMP-2 and its activator, MT1-MMP, and consequent activation of MMP-2.

MATERIAL AND METHODS

Tissues

Human brain glioma tissues and non-neoplastic brain tissues were obtained immediately after surgical removal or autopsy, fresh-frozen in liquid nitrogen and stored at -80°C for RNA and protein extraction. At the same time portions of the tissues were embedded in OCT compound (Miles, Elkhard, IN), frozen in liquid nitrogen, cut into cryostat sections, and stored at -40 √C for immunostaining. Standard light microscopic evaluation of each section stained with hematoxylin-eosin was performed for histological diagnosis. The histological grading of gliomas was determined according to the WHO standards (Kleihues et al., 1993).

Cell culture

MGM-1 was established from a human primary GBM (Moriyama et al., 1997), and brain-derived fibroblasts, MBT-3, were obtained from a metastasis to the cerebellum from a lung adenocarcinoma. Human GBM cell lines, YKG-1, A172, and T98G and human brain-derived normal fibroblast FLOW3000 were purchased from Human Science Research Resources Bank (Osaka, Japan). Another human GBM cell line U251 and a human bladder-cancer cell line T24 were obtained from the RIKEN cell bank (Tsukuba, Japan). T24 cell extracts were used as positive control in immunoblotting since their EMMPRIN expression was previously demonstrated (Muraoka et al., 1993). All these cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin G and 100 g/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37 √C.

RNA isolation

Total RNA was extracted from tissues using Trizol (Gibco BRL, Gaithersburg, MD), and poly(A)$^+$RNA was selected with an oligo-dT-Latex (Takara, Shiga, Japan) according to the manufacturer's instructions. Poly(A)$^+$RNA isolation from cultured cells was done using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA), and genomic DNA was isolated using the DnaQuick DNA extraction kit (Dainippon Seiyaku, Osaka, Japan). Commercially available human whole brain

poly(A)$^+$RNA (Clonetech, Palo Alto, CA) was also used.

RT-PCR

Poly(A)$^+$RNA (0.1 μg) was reverse transcribed by Superscript reverse transcriptase (Gibco BRL) using an oligo dT primer to prepare cDNA, according to the manufacturer's instructions. The resultant cDNAs were suspended into an aliquot containing 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM dNTPs and 7 mM anti-Taq polymerase monoclonal antibody (Clontech). In addition, each sample contained 0.1 pmol of both forward and reverse primers, and 2.5 U of Taq polymerase (Takara). PCR was carried out in a thermocycler (Perkin Elmer Cetus, Norwalk, CT) using 5 cycles consisting of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min and 15 sec, followed by 30 cycles consisting of 94°C for 10 sec, 58°C for 30 sec and 72°C for 1 min and 15 sec. Products were analyzed by 2.0% agarose gel electrophoresis.

PCR primers

Oligonucleotide primer pairs used in this study are listed in Table 1. Each pair spanned intron-exon splice sites. The sequences of the forward primers for EMMPRIN, P1 and P2 sense, correspond to bases 67 - 83 and 502 - 521, respectively, and the sequences of reverse primers for EMMPRIN, P1 and P2 antisense, correspond to bases 251 - 270 and 782 - 801, respectively, of the EMMPRIN sequence (Biswas et al., 1995). Amplification of the correct sequences of EMMPRIN was confirmed by the size of PCR product being equal to the expected size and also by nested PCR. Primer pairs for MMP-1 and MMP-3 were chosen according to Ishiguro et al. (1996) and a pair for MMP-2 according to Onisto et al. (1995).

Immunohistochemistry

Immunohistochemical staining of frozen sections was performed as described (Muraoka et al., 1993) with minor modifications. Briefly, frozen tissue sections were fixed in acetone at -40 √C for 5 min. After endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide in methanol for 30 min, sections were incubated with normal rabbit serum (1:5, DAKO, Glostrup, Denmark) for 1 hr at 37 √C. After blocking with normal serum, the sections were incubated with anti-EMMPRIN monoclonal antibody (mAb) (E11F4) (Ellis et al., 1989) overnight at 4 √C. The sections were then washed in PBS, and incubated with biotinylated anti-mouse IgG (1:10, DAKO) for 10 min at room temperature, followed by streptavidin conjugated to horseradish peroxidase (DAKO) for 10 min. The reaction was revealed with Metal-3,3'-diaminobenzidine (DAB) (Pierce, Rockford, IL) and counterstained with Mayer's hematoxylin.

Northern blot analysis

Total RNA (30 μg of each) or Poly(A)$^+$RNA (5 μg of each) was electrophoresed on 1% formaldehyde agarose gel and transblotted onto Hybond-N+ nylon membrane (Amersham, Aylesbury, U.K.) and RNA was UV-crosslinked onto the membrane. Hybridization was performed in mixed solution of 50% formamide, 5x Denhardt's solution, 25 mM phosphate buffer (pH 6.5), 0.1% SDS, 100 μg/ml of sonicated and heat-denatured salmon sperm DNA, and 5x standard saline-citrate (SSC) at 42 °C for 16 h. The blots were washed as follows: 3 times in 0.1% SDS in 1x SSC for 15 min at room

temperature; and twice in the same solution for 20 min at 65 °C. The membranes were autoradiographed with Kodak XR-5 film at -80 °C for 6 h or 18 h. The full EMMPRIN cDNA (1.6-kb) (Biswas et al., 1995) was used as a probe. For internal control of loading, the blots were subsequently hybridized to a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) probe (Clontech). The probes were radiolabeled by random priming with ^{32}P-CTP.

Co-culture experiments

Co-culture experiments were done as previously described (Nabeshima *et al.*, 1994). Briefly, cultures containing either MBT3 fibroblasts, U251 GBM cells or both, were established in 20 mm diameter wells containing 1.0 ml growth medium. A fixed number (1.0×10^5) of fibroblasts was incubated with increasing numbers (0.1, 0.5 and 1.0×10^5) of U251 cells. The cells were allowed to attach for 24 h at 37 √C in a humid atmosphere of 5% CO_2 and 95% air, after which their media were replaced with fresh serum-free DMEM containing 0.2% lactalbumin hydrolysate (0.5 ml/well) prior to beginning the experiment. Each experimental condition was done in duplicate wells. Culture fluids were replaced with fresh serum-free DMEM at 3 days, and harvested at 6 days. The harvested media were used for zymography and immunoblotting.

For the co-culture experiments with E11F4, U251 cells were preincubated with E11F4 before coculturing with fibroblasts. Typically $0.1\text{-}0.25 \times 10^5$ U251 cells were incubated at 37 √C for 45 min with 200 µl of E11F4, after which the whole mixture was added to the fibroblasts (1×10^5) and allowed to attach for 24 h at 37 √C as described above. Then, their media were replaced with serum-free DMEM containing varying amounts of serum-free E11F4. This replacement was done again at 3 days, and culture fluids were harvested at 6 days.

To detect MT1-MMP production in the coculture experiments, the cells were harvested at 6 days and lysed by boiling for 5 min in 2% SDS, 0.1 M dithiothreithol/1 M Tris-HCl (pH 6.8), followed by centrifugation at 11,000*g* for 5 min. These SDS extracts were subjected to immunoblotting.

Zymography

Gelatinolytic activities in each conditioned medium were demonstrated using gelatin (1 mg/ml) as a substrate as described (Heussen and Dowdle, 1980). SDS-PAGE was performed under non-reducing conditions using a 9% separating gel containing 1 mg/ml gelatin. After electrophoresis, the gel was shaken gently in detergent buffer (5 mM $CaCl_2$/2.5% Triton-X 100/50 mM Tris-HCl, pH 7.6) at room temperature for 60 min to remove SDS, and then incubated in reaction buffer (0.15 M NaCl/10 mM $CaCl_2$/0.02% NaN_3/50 mM Tris-HCl, pH 7.6) at 37 √C for 40 h followed by staining with 2.5% Coomassie brilliant blue in 30% methanol and 10% acetate. Enzyme activity was detected as a clear band on the resulting blue background of undigested gelatin, and their photos were subjected to image analysis (Adobe Photoshop, Adobe Systems, Mountain View, CA).

Preparation and extraction of membranes

Extraction of EMMPRIN from glioblastoma-cell membranes were performed as described by Ellis et al. (1989). Tumor cells were harvested from confluent cultures in 25-cm^2 culture bottles by mechanical scraping and were then suspended and sonicated in 50 mM Tris-HCl (pH 7.4)/0.24 M sucrose. Tissue samples were homogenized in a Polytron in the same Tris-sucrose buffer prior to sonication. The sonicated cell suspension and tissue homogenate were centrifuged at 500*g* for 1 h. The

membrane pellet was extracted with 0.15 M NaCl/0.5% Nonidet P-40/1 mM EDTA (Nakarai, Kyoto, Japan)/2 mM phenylmethylsulfonyl fluoride (Boehringer Mannheim, Germany)/10 mM Tris-HCl (pH 8.2), at 4 √C overnight. The extract was centrifuged at 100,000*g* for 1 h and the supernatant was kept at 4 √C until use. The presence of EMMPRIN in the membrane extract was assayed using immunoblotting.

Immunoblotting

SDS-PAGE of tissue and cell extracts and conditioned medium was performed under reducing conditions using a 5-15% gradient separating gel (Biomate, Tokyo, Japan). After electrophoresis, the proteins were transferred electrophoretically to Immobilon membrane (Millipore, Bedford, MA). After the non-specific sites were blocked with 5% non-fat dry milk in 0.05% Tween-20/Tris-buffered saline, pH 7.6 (TBS-T), at 37 √C for 3 h, the membrane was incubated overnight at 4 √C with mouse mAb to human EMMPRIN (E11F4), human MMP-2 (75-7F7, Fuji Chemical Industries, Takaoka, Japan) or MT1-MMP (114-1F2, Fuji Chemical Industries) dissolved in TBS-T containing 1% BSA. The membrane was washed three times with TBS-T and was incubated for 1 h with peroxidase-conjugated goat anti-mouse IgG. The color was developed with chemiluminescence reagents (DuPont NEN, Boston, MA) according to the manufacturer's instructions. The bands on the film were subjected to image analysis (Adobe Photoshop).

RESULTS

Detection of EMMPRIN and MMP-1, 2 and 3 expression in brain tissues by RT-PCR

First, using RT-PCR, we examined expression of EMMPRIN and EMMPRIN-inducible MMPs, MMP-1, 2 and 3, in normal brain and glioma tissues. The results are shown in Fig.1 and summarized in Table 2. EMMPRIN was detected in all cases of both non-neoplastic brain and glioma. Both primer pairs (P1 and P2) yielded products of the expected sizes. On the other hand, MMP-2 was clearly detected in all the GBM tissues, but was not detected in any of non-neoplastic brain tissues. Less frequent expression compared with GBM was noted in low grade gliomas, such as pilocytic astrocytoma and astrocytoma. Expression of MMP-1 and 3 was detected in some of both non-neoplastic and glioma tissues without any obvious pattern.

Immunohistochemical localization of EMMPRIN

Since EMMPRIN expression was detected by RT-PCR in both non-neoplastic and glioma tissues in the brain, we examined immunohistochemical localization of EMMPRIN in these tissues. In non-neoplastic portions of the brain, EMMPRIN immunostaining was demonstrated only in vascular endothelial cells (Fig. 2A), whereas in GBM it was tumor cells that were stained positive, and proliferating blood vessels were negative (Fig. 2C). The tumor cells showed strong positivity with accentuation along the cell membrane. GBM cells at the invasion front were also positive for EMMPRIN (Fig. 2D), but in less cellular low grade astrocytoma regions at the periphery of anaplastic astrocytoma or GBM, tumor cells stained negative, with only vascular endothelial cells being positive (Fig. 2B). The latter regions did not show Gd-enhancement on magnetic resonance imaging (MRI) (data not shown), suggesting the presence of normally functioning BBB. GBM portions with EMMPRIN-negative proliferating blood vessels demonstrated intense Gd-enhancement, indicating loss

of normal BBB function.

Expression of EMMPRIN mRNA and protein in non-neoplastic and glioma tissues

Since localization of EMMPRIN was quite different between non-neoplastic brain tissue and gliomas, we next investigated the level of mRNA specific for EMMPRIN in these tissues by Northern blot analysis. All the samples examined expressed EMMPRIN transcripts of ~1.7 kb, although the intensity of the signals varied significantly according to the different sources (Fig. 3). The relative abundance of EMMPRIN mRNA, expressed as a ratio of the radioactivity of the 1.7 kb EMMPRIN band to that of the corresponding G3PDH band, was 0.02 +/- 0.004 (n=5) for non-neoplastic brain tissue, 0.061 +/- 0.01 (n=3) for low-grade astrocytic tumors (pilocytic astrocytoma and astrocytoma), 0.146 +/- 0.03 (n=5) for anaplastic astrocytoma, and 0.335 +/- 0.06 (n=12) for GBM. Thus, EMMPRIN mRNA was obviously expressed at higher levels in astrocytic tumors than in non-neoplastic tissue. Moreover, even in the astrocytic tumors, expression levels increased as the grade of malignancy went up: EMMPRIN mRNA was expressed 2.4 and 5.5 times more in anaplastic astrocytoma and GBM, respectively, compared with low grade astrocytic tumors.

The immunoblot of tissues with E11F4 in each case revealed distinct immunoreactivity as a doublet at 42 and 40 kDa (Fig. 4). T24 bladder carcinoma cell extracts, used as a positive control, showed a broad band around 50 kDa as described (Muraoka et al., 1993). EMMPRIN protein was also expressed as a doublet in LX-1 human lung carcinoma cells, but the molecular weights in this case were 58 and 54 kDa (Nabeshima et al., 1991). These differences in molecular weight of EMMPRIN are mainly due to varying extents of glycosylation since the protein backbone of EMMPRIN corresponds to an approximate molecular weight of 27 kDa (Biswas et al., 1995). Although previous results suggest that glycosylation may be important for functional activity (Guo et al., 1997), the significance of differing extents of glycosylation is not known. Semiquantitative analysis of the immunoreactive bands in Fig. 4 revealed that EMMPRIN was expressed approximately 2.7 times more at the protein level in astrocytic tumors than in non-neoplastic brain tissues. Correlation of the protein expression levels with tumor progression could not be determined since only one low grade astrocytoma tissue was available for this assay.

EMMPRIN expression in glioblastoma cell lines

We investigated whether human glioblastoma cell lines also expressed EMMPRIN. EMMPRIN expression was demonstrated by RT-PCR (30 cycles) in all the glioblastoma cell lines examined (Fig. 5A). However, levels of EMMPRIN protein in membrane extracts from the cells were quite different among the different lines (Fig. 5B). The highest level of expression was observed as a broad band around 50 kDa in U251 cells, whereas a protein band could barely be detected in YKG-1 and the other lines expressed intermediate levels. Therefore we used the U251 cell line for the coculture experiments described below. EMMPRIN protein was not detected in MBT-3 fibroblasts taken from adult brain, and FLOW3000 fibroblasts obtained from the embryonal brain showed a faint band at approximately 50 kDa.

Stimulation of MMP-2 production and activation by glioblastoma cell EMMPRIN in cocultures of glioma cells and fibroblasts

To assess the potential stimulatory effect of glioblastoma-expressed EMMPRIN on MMP

production by fibroblasts *in vitro*, we ran coculture experiments. A fixed number (1×10^5) of MBT-3 or FLOW3000 brain-derived fibroblasts was incubated with increasing numbers (0.1 to 1.0×10^5) of U251 glioblastoma cells. Fig. 6A shows gelatin zymography of the conditioned media harvested on day 6 of coculture of U251 glioblastoma cells with MBT-3 fibroblasts. Both MBT-3 and U251 cells exhibited gelatinolytic bands at 68 and 60 kDa. The 68-kDa band corresponds to the molecular mass of the proform of MMP-2 in the absence of dithiothreitol and the 60-kDa band corresponds to the activated form (Overall and Sodek, 1990). These gelatinolytic activities corresponding to pro- and activated MMP-2 were both stimulated in cocultures compared to individual cultures of each cell type. Moreover, coculturing caused a far greater increase in the activated form of MMP-2 than the proform. Similar results were also obtained in cocultures of U251 tumor cells with FLOW3000 fibroblasts, although the stimulatory effect was a little less than in cocultures of U251 and MBT-3 cells (data not shown).

To confirm the involvement of EMMPRIN in stimulation of MMP-2 activity in cocultures, we performed the coculture experiment in the presence of varying concentrations of activity-blocking anti-EMMPRIN mAb, E11F4. Fig. 6B shows an immunoblot with anti-MMP-2 mAb of the conditioned media harvested on day 6 of culture; only pro-MMP-2 was demonstrated with this antibody. The immunoblots confirmed that MMP-2 production was stimulated in cocultures compared to individual cultures of MBT-3 and U251 cells. This stimulation was inhibited by E11F4 in a dose-dependent manner, while IgG control did not cause any inhibition.

Stimulation of MT1-MMP production by glioblastoma cell EMMPRIN in cocultures

Since the activated form of MMP-2 was greatly stimulated by EMMPRIN in the cocultures, we examined whether EMMPRIN also stimulates production of the activator of MMP-2, MT1-MMP. Fig. 7 shows an immunoblot with anti-MT1-MMP mAb of extracts of cells harvested on day 6 of culture. As expected, the level of MT1-MMP was stimulated in cocultures compared to control, i.e. the combined extracts of equivalent numbers of MBT-3 and U251 cells after they had been cultured separately. This stimulatory effect was inhibited by E11F4 in a dose-dependent manner (Fig. 7).

DISCUSSION

In this study, we have demonstrated that expression levels of EMMPRIN mRNA and protein are higher in astrocytic tumors, especially in malignant ones, than in non-neoplastic brain tissues, and that EMMPRIN is clearly localized to malignant astrocytic tumor cells. Moreover, *in vitro,* EMMPRIN on glioma cells was shown to stimulate production of pro-MMP-2 and MT1-MMP and consequently the production of activated MMP-2. MMP-2 expression correlates with the malignant progression of gliomas *in vivo* (Nakano et al., 1995; Sawaya et al., 1996; Lampert et al., 1998), and MT1-MMP mRNA levels are significantly higher in malignant astrocytomas than in low-grade gliomas and normal brain tissues (Yamamoto et al., 1996; Lampert et al., 1998). Thus, it is possible that EMMPRIN expressed on glioma cells may be involved in gliomal invasion via stimulating stromal cell production of MMP-2 and its activation. In support of this proposition, it has been shown recently in other cancer types that stromal production of MMP-2 promotes tumor progression (Itoh et al., 1998). Also, since EMMPRIN mRNA expression correlated with malignant progression of gliomas in this study,

EMMPRIN may be responsible for the increased expression of MMP-2 and MT1-MMP in malignant gliomas. Whether the levels of EMMPRIN mRNA in low grade astrocytic tumors is an indicator of how fast progression of a given tumor will occur is an interesting topic now under investigation in our laboratories.

In the brain, the distribution of ECM proteins that are known substrates for MMPs, such as laminin, type IV collagen, fibronectin and vitronectin, is limited to vascular basement membranes (BMs) and the glial limitans externa, which represents another true BM that covers the cortical surface and separates the astrocytic foot processes from the subarachnoid space and pial cells (Giese and Westphal, 1996). In the intracortical perivascular space, called the Virchow-Robin space, the BM separates into two parts. The endothelial part surrounds the vessel, and beneath the pial cells, in contact with astrocytic foot processes, is the glial limitans externa (Giese and Westphal, 1996). During glioma cell infiltration, the white matter fiber pathways are the predominant route of tumor spread, while perivascular and subpial spread along the BM structure described above is another route (Burger and Sheithauer, 1994). However, penetration of BM and consequent invasion into the vessel lumen and the subarachnoidal space rarely occur *in vivo*. In this light, malignant glioma cells may require modification of the surface structures of vascular BMs and the glial limitans externa such that they become more favorable for tumor spread rather than a mechanism for penetration of these structures. Thus MMPs may remodel the ECM at these sites in such a way as to promote cell adhesion and migration rather than penetration. Blood vessels in the cerebral cortex, both in adults and infants, contain fibroblasts outside the endothelium (Zhang et al., 1997), and these fibroblasts take part in the formation of granulation tissue together with capillaries in response to tissue injuries (Gray and Nordmann, 1997). Thus it is possible that MMP production by these fibroblasts is involved in perivascular spread of glioma cells as a result of stimulation by EMMPRIN expressed on the glioma cells.

A second mechanism whereby gliomal cell EMMPRIN might influence tumor progression is via stimulation of angiogenesis, a characteristic feature of GBM. MMP-2 (Itoh et al., 1998) and MT1-MMP (Hiraoka et al., 1998) have been implicated in angiogenesis. Thus EMMPRIN-stimulated production of these enzymes by fibroblasts in perivascular regions may contribute to tumor angiogenesis. In addition, it has been shown recently that EMMPRIN stimulates endothelial cell production of several MMPs (S.Zucker, M.Drews, C.Conner, J.Cao & B.Toole, ms in preparation) which may be directly involved in endothelial cell migration during tumor angiogenesis.

Immunohistochemical studies have demonstrated that EMMPRIN is distributed diffusely throughout human bladder, breast and lung carcinomas, with adjacent normal tissue being mainly negative (Muraoka et al., 1993; Polette et al., 1997). In our study, malignant glioma cells, such as GBM and anaplastic astrocytoma cells, also showed diffuse immunoreactivity. This diffuse immunoreactivity within the tumor suggests that EMMPRIN may be useful as an immunohistochemical tumor marker. For example, EMMPRIN immunohistochemistry may help to detect infiltrating malignant astrocytic tumor cells. This possibility is now being investigated in our laboratories.

In non-neoplastic portions of the brain, immunostaining of EMMPRIN is confined to the vascular endothelial cells. Moreover, EMMPRIN-positive endothelial cells are confined to the brain: EMMPRIN could not be detected in vascular endothelial cells by immunostaining of cryostat sections of various tissues outside the brain as far as we have examined (K.Nabeshima, T.Sameshima, S.Sato, B.P.Toole, H.Kataoka & M.Koono, unpublished data). This immunolocalization is very similar to that

of HT7/neurothelin, the species homologue of EMMPRIN in the chicken: HT7/neurothelin is exclusively expressed on endothelial cells of the central nervous system but not on systemic endothelial cells (Seulberger et al., 1990; Schlosshauer and Herzog, 1990). The molecular weight of HT7/neurothelin is approximately 43 kDa (Schlosshauer and Herzog, 1990), which is almost the same as that of EMMPRIN expressed in human brain and gliomas in our study. Endothelial cells in the circumventricular organs that lack a blood-brain barrier, such as the pituitary, median eminence, subfornical organ, pineal gland and area postrema, were negative for HT7/neurothelin (Albrecht et al., 1990). These observations suggest a correlation between blood-brain barrier function and expression of HT7/neurothelin. We also found that, in the case of malignant gliomas, endothelial cells obtained from intratumoral areas showing Gd-enhancement on MRI, which indicates loss of blood-brain barrier function, are negative for EMMPRIN, whereas those from the peritumoral area showing no Gd-enhancement are EMMPRIN-positive (unpublished data). Thus, in the normal human brain, EMMPRIN may also be closely related to blood-brain barrier function, but its specific function in this system is not yet understood. Once EMMPRIN is over-expressed aberrantly in glioma cells, however, it may cause exaggerated remodeling of ECM via interaction with fibroblasts and endothelial cells, which consequently facilitates tumor cell infiltration and tumor angiogenesis. Contact of EMMPRIN-expressing cells with stromal cells might be a key event, since normal EMMPRIN-expressing endothelial cells are separated from the outer stromal cells by the basement membrane.

Our study suggests that interruption of tumor cell-fibroblast (or other stromal cell) interaction could be a target of anti-invasion therapy in gliomas. Demonstration of the involvement, *in vivo*, of EMMPRIN in the stimulation of production of MMPs essential to glioma progression would be necessary to confirm this possibility. This is now under investigation in our laboratories.

Acknowledgements

This work was supported in part by a Grant-in-Aid for encouragement of Young Scientists from the Ministry of Education, Science and Culture of Japan (No. 05770157) and grants from the Special Coordination Funds of the Science and Technology Agency of Japan and the U.S. Army (DAMD 17-95-1-5017).

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Figure legends

Figure 1. RT-PCR for EMMPRIN, and MMP-1, 2 and 3. Poly(A)$^+$RNAs prepared from non-neoplastic brain (NB), pilocytic astrocytoma (PA), low grade astrocytoma (A), anaplastic astrocytoma (AA) and glioblastoma multiforme (GBM) tissues were used for the reaction. The 204 and 300 bp products of EMMPRIN were generated with different combination of primers (P1 sense/P1 antisense and P2 sense/P2 antisense, respectively).

Figure 2. Immunohistochemical demonstration of EMMPRIN protein in normal (A) and glioma (B-D) tissues. (A) In the normal brain, vascular endothelial cells (arrows) show strong positive staining along the lumen, while astrocytes stain negatively. (B) At the periphery of anaplastic astrocytoma where histology corresponds to that of low grade astrocytoma with low cellularity and no mitotic figures, tumor cells stain negatively, with vascular endothelial cells (arrows) being positive. (C) In the center of GBM, tumor cells show diffuse cytoplastic staining with accentuation along the cell membrane, whereas proliferating blood vessels with thickened layers of cells stain negatively. (D) The invasion front of GBM consists of infiltrating EMMPRIN-positive, pleomorphic tumor cells. x120.

Figure 3. Northern-blot analysis of EMMPRIN mRNA in human non-neoplastic brain and glioma tissues. Poly (A)$^+$ RNA (5 μg) from each cell line was applied to the gel, then the blot was probed sequentially with EMMPRIN cDNA (indicated by "EMMPRIN") and G3PDH cDNA (indicated by "G3PDH") and autoradiographed for 6 h. Lanes 1-5, non-neoplastic brain; lane 6, pilocytic astrocytoma; lanes 7-8, low grade astrocytoma; lanes 9-13, anaplastic astrocytoma; and lanes 14-25, GBM.

Figure 4. Immunoblot analysis of EMMPRIN protein expressed in non-neoplastic and neoplastic brain tissues. Cell and tissue extracts were subjected to immunoblotting with mAb (E11F4) to EMMPRIN. Lane 1, cultured T24 urinary bladder carcinoma cells as positive control; lanes 2-4, non-neoplastic brain; lane 5, low grade astrocytoma; lane 6, anaplastic astrocytoma; lanes 7-10, GBM.

Figure 5. (A) Detection of EMMPRIN mRNA expression in GBM cell lines by RT-PCR. Lane 1, U251; lane 2, YKG-1, lane 3, A172; lane 4, T98G; lane 5, MGM1. (B) Immunoblotting of cell extracts with mAb (E11F4) to EMMPRIN. Lane 1, T24 as positive control; lane 2, U251; lane 3, YKG-1; lane 4, A172; lane 5, T98G; lane 6, MGM1; lane 7, MBT-3 fibroblasts; lane 8, FLOW3000 fibroblasts.

Figure 6. (A) Stimulation of MMP-2 gelatinolytic activity in cocultures of U251 glioblastoma cells and MBT-3 fibroblasts. Culture media collected at day 6 of culture were subjected to zymography. Lane 1, MBT-3 alone; lane 2, U251 alone (0.1×10^5); lane 3, U251 alone (0.5×10^5); lane 4, U251 alone (1.0×10^5); lane 5, MBT-3 and U251 (1:0.1); lane 6, MBT-3 and U251 (1:0.5); and lane 7, MBT-3 and U251 (1:1). (B) Inhibition of stimulated production of MMP-2 in cocultures of U251 and MBT-3 cells by anti-EMMPRIN mAb treatment. Culture media collected at day 6 of culture were subjected to immunoblotting with anti-MMP-2 mAb. Lane 1, MBT-3 alone; lane 2, U251 alone (0.1×10^5); lane 3, MBT-3 and U251 (1:0.1); lane 4, MBT-3 and U251 (1:0.1) with 100 μl anti-EMMPRIN mAb (E11F4) in a total volume of 500 μl (100/500); lane 5, MBT-3 and U251 (1:0.1) with E11F4 (200/500); lane 6, MBT-3 and U251 (1:0.1) with E11F4 (400/500); lane 7, MBT-3 and U251 (1:0.1) with IgG (400/500).

Figure 7. EMMPRIN-dependent stimulation of MT1-MMP production in cocultures of U251 glioblastoma cells and MBT-3 fibroblasts. Extracts of cells collected at day 6 of culture were subjected to immunoblotting with anti-MT1-MMP mAb. Lane 1, MBT-3 (1.0×10^5) and U251 (0.25×10^5) cultured separately and extracted together; lane 2, cocultured MBT-3 and U251 (1:0.25); lane 3, cocultured MBT-3 and U251 (1:0.25) with 100 μl anti-EMMPRIN mAb (E11F4) in a total volume of 500 μl (100/500); lane 4, cocultured MBT-3 and U251 (1:0.25) with E11F4 (200/500); lane 5, cocultured MBT-3 and U251 (1:0.25) with E11F4 (400/500); lane 6, cocultured MBT-3 and U251 (1:0.25) with IgG (400/500).

Table 1. Sequences of PCR primers sets for EMMPRIN and MMPs

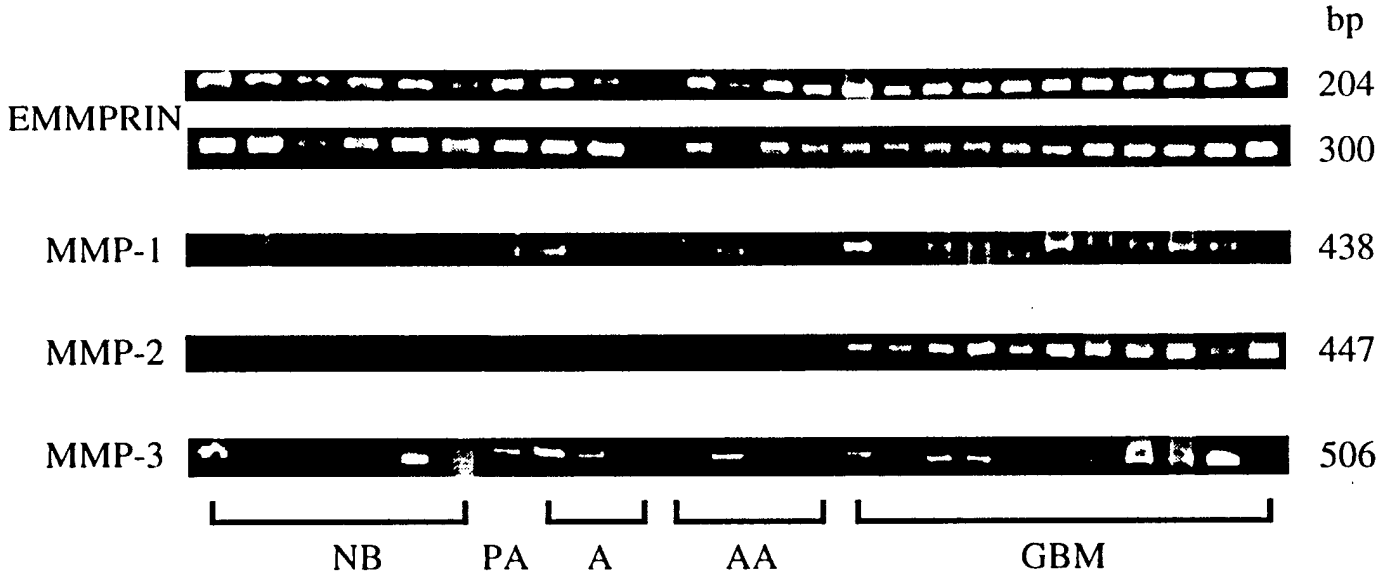
cDNA		primer sequence (5'-3')	product
EMMPRIN	P1 sense	5'-GCCGGCACAGTCTTCACTAC-3'	204 bp
	antisense	5'-GAGGAAGACGCAGGAGTACT-3'	
	P2 sense	5'-GAGCTACACATTGAGAACCT-3'	300 bp
	antisense	5'-GTTCCTCTGGCGGACGTTCT-3'	
MMP-1	sense	5'-GGTGATGAAGCAGCCCAG-3'	438 bp
	antisense	5'-CAGTAGAATGGGAGAGTC-3'	
MMP-2	sense	5'-ACCTGGATGCCGTCGTGGAC-3'	447 bp
	antisense	5'-TGTGGCAGCACCAGGGCAGC-3'	
MMP-3	sense	5'-GAGTCAGGTCTGTGAGTGAGTG-3'	506 bp
	antisense	5'-ACTCCGACACTCTGGAGGTGAT-3'	

MMP-1, interstitial collagenase; MMP-2, gelatinase A; MMP-3, stromelysin-1

Table 2. RT-PCR analysis of EMMPRIN and MMP expression in non-neoplastic brain and gliomas

expressed mRNA	positive cases				
	NB	PA	A	AA	GBM
EMMPRIN	6/6	1/1	3/3	4/4	11/11
MMP-1	2/6	1/1	2/3	3/4	9/11
MMP-2	0/6	1/1	1/3	2/4	11/11
MMP-3	4/6	1/1	2/3	2/4	9/11

NB, non-neoplastic brain tissue; PA, pilocytic astrocytoma; A, astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma multiforme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MMP-1
438
MMP-2
447
MMP-3
506
NB
PA
A
AA
GBM

Figure 2

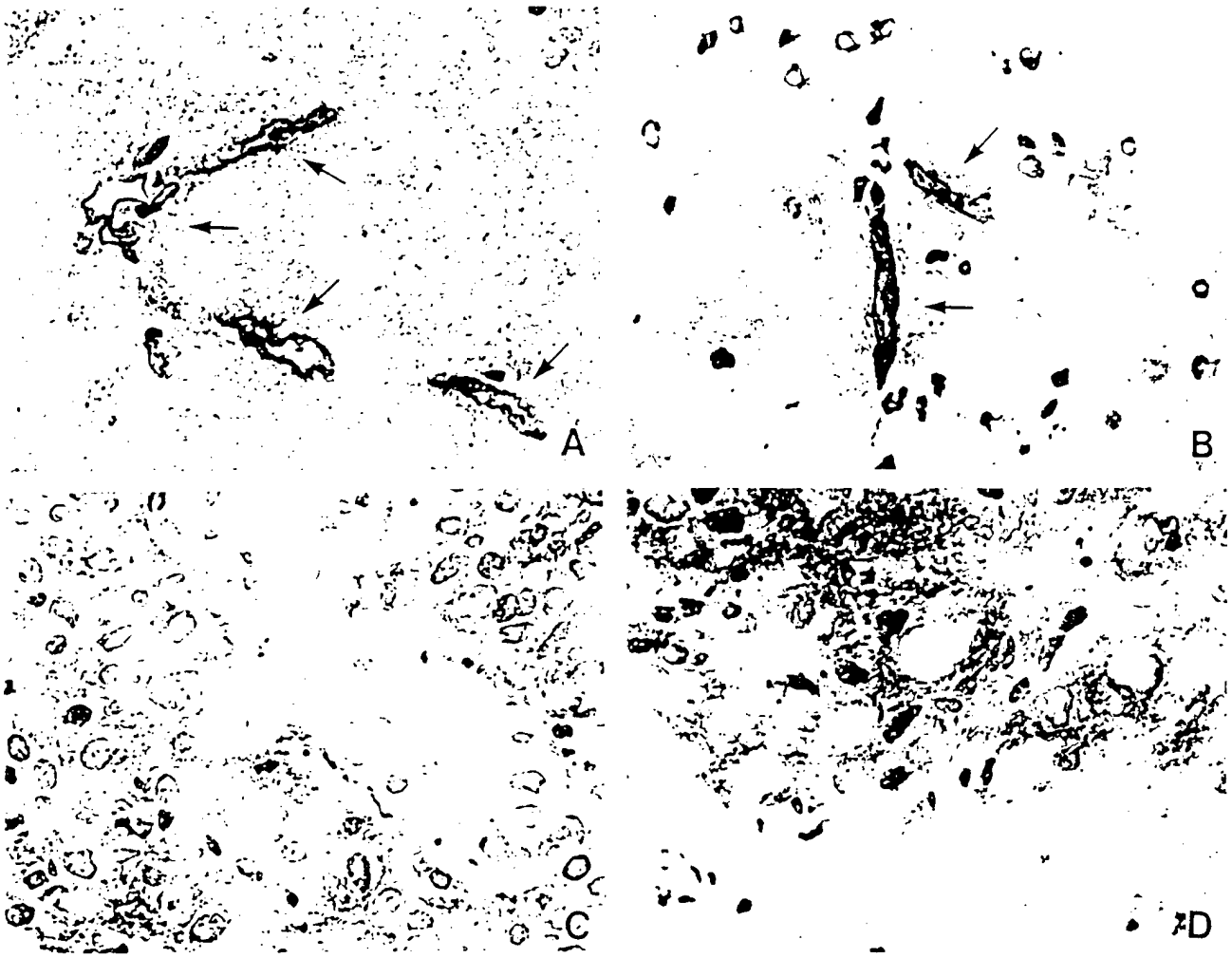

Figure 3

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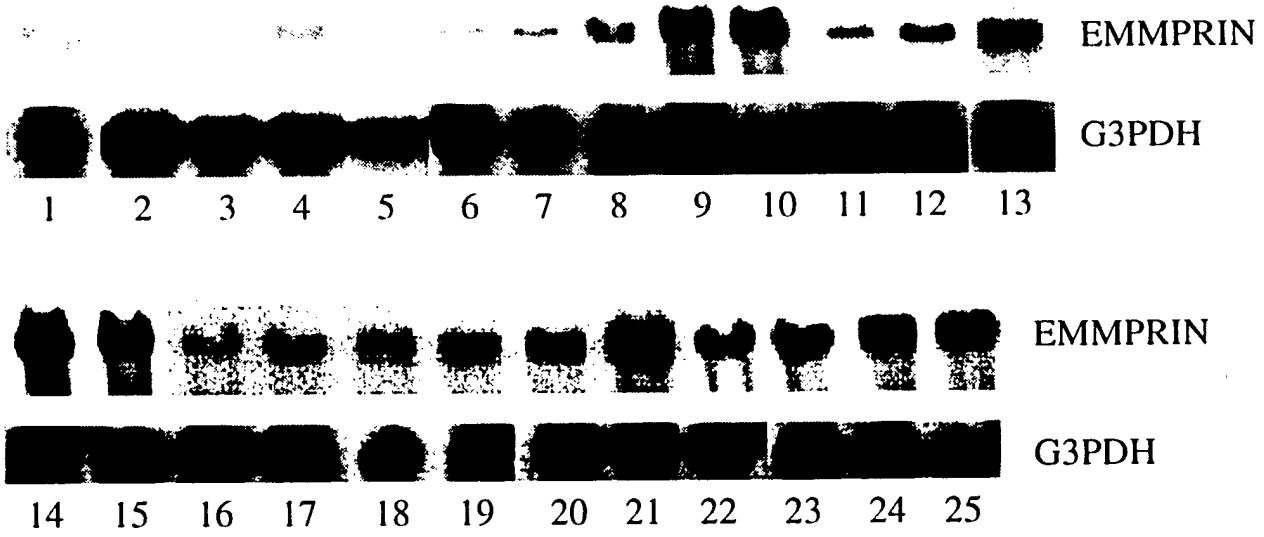

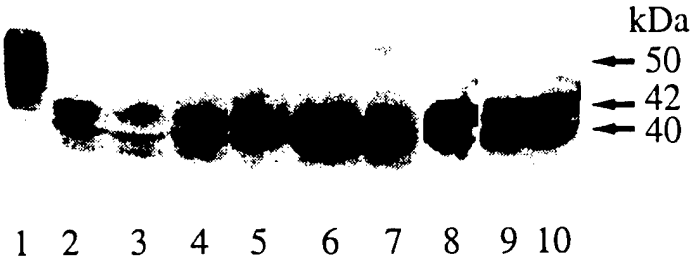
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40
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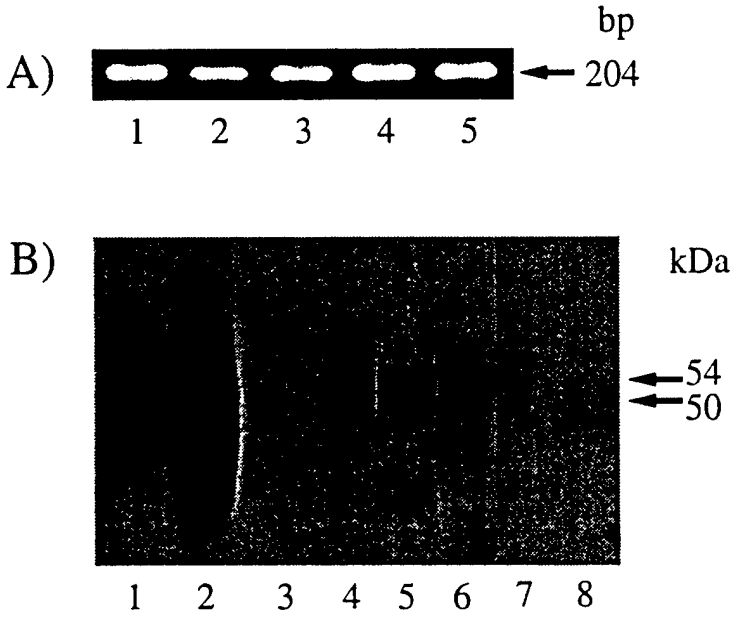
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kDa
54
50
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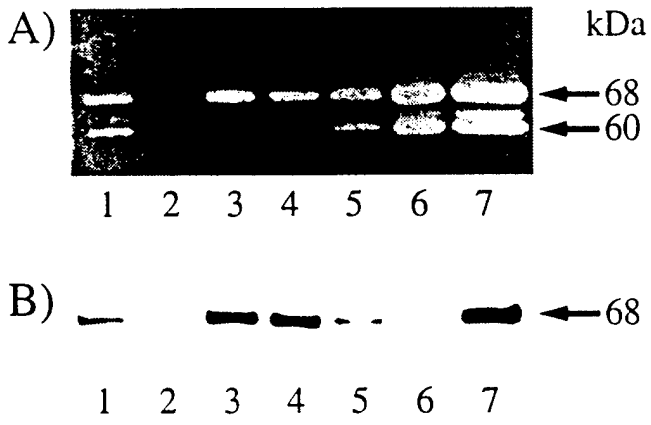
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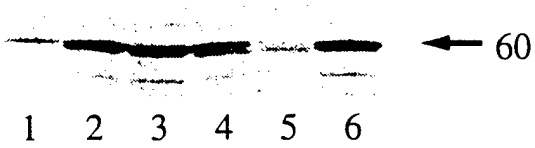
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